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CHOLINESTERASES IN SENILE DEMENTIA OF ALZHEIMER-TYPE

A thesis submitted to the University of Newcastle upon Tyne

for the Degree of Doctor of Philosophy

by

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Department of Pathology

September, 1984

Dedicated to Sue

"A man would do nothing if he waited until  
he could do it so well that no one would  
find fault with what he has done"

Cardinal Newman

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#### Cross references

Cross references are included throughout this thesis and when referring to a section in a different chapter are written in full (for example, chapter 1, section II.4.b). Cross references to different sections within the same chapter or sub-section omit the chapter number and refer only to the section of interest (for example, section II.4.b or section 4.b respectively).

## PUBLICATIONS

Some of the results described in the present thesis have been published, submitted or presented elsewhere in the following:

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## ABBREVIATIONS

Abbreviations used in this thesis include:

$A_4$ ,  $A_8$ ,  $A_{12}$  = asymmetric molecular forms of acetylcholinesterase consisting of 4, 8 and 12 catalytically active subunits respectively

ACh = acetylcholine

AChE = acetylcholinesterase

ANOVA = analysis of variance

$BC_1$ ,  $BC_2$  and  $BC_3$  = electrophoretically separated forms of plasma butyrylcholinesterase

BChE = butyrylcholinesterase

BW284c51 = 1:5-bis (4-allyldimethylammonium phenyl) pentan-3-one dibromide

ChAT = choline acetyltransferase

CNS = central nervous system

CSF = cerebrospinal fluid

DA = dopamine

DBH = dopamine- $\beta$ -hydroxylase

DTNB = 5,5-dithiobis-2-nitrobenzoic acid

EDTA = ethylenediaminetetraacetic acid

EGTA = ethyleneglycol-bis-( $\beta$ -aminoethyl ether)-N,N,N,N'-tetraacetic acid

$G_1$ ,  $G_2$  and  $G_4$  = globular molecular forms of acetylcholinesterase consisting of 1, 2 and 4 catalytically active subunits respectively

GABA =  $\gamma$ -amino butyric acid

GAD = glutamic acid decarboxylase

5-HIAA = 5-hydroxyindoleacetic acid

HACUP = high-affinity choline uptake

HVA = homovanillic acid

iso-OMPA = tetraisopropylpyrophosphoramidate

$K_m$  = Michaelis constant

MHPG = monohydroxyphenylglycol

NA = noradrenaline

NaCl = sodium chloride

NAD = nicotinamide adenine dinucleotide

nbM = nucleus basalis of Meynert

ONPG = O-nitrophenyl- $\beta$ -D-galactopyranoside

pseudoChE = pseudocholinesterase

S = Svedberg unit (sedimentation velocity)

SDAT = senile dementia of the Alzheimer type

SLI = somatostatin-like immunoreactivity

TEMED = N,N,N',N'-tetramethylethylenediamine

Tris = tris(hydroxymethyl)-aminomethane

ABSTRACT

Aspects of cholinesterase distribution were studied in both the central nervous system (CNS) and blood in relation to the central cholinergic deficit in senile dementia of the Alzheimer type (SDAT). In the SDAT neocortex, the greatest loss of cholinergic-related acetylcholinesterase (AChE) activity was from the upper cortical layers. Previous reports indicate that the majority of neuropathological, neurochemical and morphological changes occur in the lower cortical layers, suggesting that the cholinergic deficit in SDAT may not be directly associated with the primary disease processes of this disorder. Furthermore, the study of AChE molecular forms revealed that in both SDAT and demented Parkinson's disease subjects, the loss of neocortical AChE activity is due to a selective loss of the  $G_4$  form of the enzyme. Hence, the cortical cholinergic changes in SDAT may not be uniquely associated with Alzheimer-type neuropathological changes. A similar extensive loss of the  $G_4$  form of AChE occurred in the cholinergically-denervated rat hippocampus, indicating that this form is probably associated with cholinergic axonal processes and suggests that these structures degenerate in demented Alzheimer-type and Parkinsonian cases.

In contrast to AChE, the physiological function of butyrylcholinesterase (BChE) is unknown. However, the different intracortical and inter-regional distributions of AChE and BChE in the CNS, along with the observation that despite reduced levels of cholinergic activity in SDAT, BChE was unaltered, all suggest that BChE is not intimately associated with central cholinergic neurotransmission.

In the blood, measurement of cholinesterase activity in SDAT revealed normal levels of erythrocyte AChE and plasma BChE and elevated plasma AChE activity. The increased plasma AChE activity may reflect increased leakage through the blood-brain barrier and/or increased release from degenerating cholinergic structures.

Whilst these results confirm the involvement of the central cholinergic system in SDAT, they also, however, suggest that these changes may be secondary to more fundamental pathological processes.



AIMS

Since acetylcholinesterase is not only involved in the biochemical cholinergic deficit in senile dementia of the Alzheimer type (SDAT), but also, along with butyrylcholinesterase, assumes particular importance in the experimental treatment of the memory deficit of SDAT with anticholinesterases, the aim of the present study was to examine various features of these enzymes in this disorder. Thus, the laminar distribution of the cholinesterases in the neocortex (chapter 2) and the involvement of the different cholinesterase molecular forms (chapter 3) were investigated in postmortem brain samples obtained from SDAT subjects. Furthermore, since cholinesterases are also found in the blood, the levels of these enzymes were measured in blood samples obtained from SDAT subjects (chapter 4) in order to evaluate the usefulness of these enzymes as possible peripheral markers of the central cholinergic deficit.

## Chapter 1:

### GENERAL INTRODUCTION

The general introduction is divided into two main parts; part I and part II. Part I, which is divided into eight sections, introduces various aspects of senile dementia of the Alzheimer-type (SDAT) and establishes the central cholinergic deficit as the major biochemical abnormality discovered to date in SDAT patients. Part II, which is divided into nine sections, deals with various aspects of cholinergic neurotransmission paying particular attention to the central cholinergic system and more specifically the cholinesterases which, in the terminology used here, includes both acetylcholinesterase and pseudocholinesterases.



## PART I

SENILE DEMENTIA OF THE ALZHEIMER TYPE AND INVOLVEMENT OF THE  
CENTRAL CHOLINERGIC SYSTEM

## 1. Dementia

Dementia has been succinctly defined as a syndrome involving a global disturbance of higher mental function in an alert patient (Marsden, 1978) and there is impairment in at least three of the following mental activities; language, memory, visuospatial skills, personality and cognition (Cummings et al, 1980). Diseases that are associated with the symptoms of dementia are numerous and of widely differing aetiologies and table 1 lists the various causes of dementia as described by Haase (1977).

Dementia may be static or progressive, and reversible or irreversible depending on its cause and treatment. The concept of treatable dementias has only recently become established and it has been estimated that 10 to 30% of dementia cases may be treatable (see Cummings, 1983). However, the most prevalent of all the dementing disorders (see section 4) is irreversible and untreatable. It is this disorder, dementia of the Alzheimer-type, that is the subject of the present thesis.

## 2. Alois Alzheimer

Alois Alzheimer (1864-1915) was a German neuropathologist who worked in close collaboration with the renowned neuropathologist, Franz Nissl. Alzheimer devoted his career to the investigation of the anatomical changes underlying dementia and in 1907 he first described "a peculiar disease of the cerebral cortex" in a 55 year old woman with memory loss and a profound personality disturbance (Alzheimer, 1907). Death had occurred four and a half years after the first clinical signs of the disease and at autopsy the brain was atrophic without macroscopic lesions. However, microscopic preparations of the cerebral cortex stained using the Bielschowsky silver method showed abnormal intracellular accumulations of fibrous material that merged into dense bundles. In addition, scattered throughout the cortex were lesions associated with the deposition of a "peculiar substance" that was very refractory to dyes. Alzheimer concluded that "in summary, we are apparently confronted with a distinctive disease process". Whilst the lesions (senile plaques) associated with the "peculiar substance" (amyloid) that was refractory to dyes had been described previously

TABLE 1

Diseases causing dementia <sup>a</sup>Diffuse Paranchymatous Diseases  
of the Central Nervous System

## So-called presenile dementias

Alzheimer's disease

Pick's disease

Kraepelin's disease

Parkinsonism-dementia

complex of Guam

Huntington's chorea

## Senile dementia

Alzheimer-type

## Other degenerative diseases

Hallervorden-Spatz disease

Spinocerebellar degenerations

Progressive myoclonus epilepsy

Progressive supranuclear palsy

Parkinson's disease

Metabolic Disorders

Myxedema

Disorders of the parathyroid  
glands

Wilson's disease

Liver disease

Hypoglycemia

Remote effects of carcinoma

Cushing's syndrome

Hypopituitarism

Uremia

Dialysis dementia

Metachromatic leukodystrophy

Vascular Disorders

Multi-infarct dementia

Inflammatory diseases of blood  
vessels

Disseminated lupus

erythematosus

Thromboangitis obliterans

Aortic arch syndrome

Binswanger's disease

Arteriovenous malformations

Hypoxia and AnoxiaNormal Pressure HydrocephalusDeficiency Diseases

Wernicke-Korsakoff syndrome

Pellegra

Marchiafava-Bignami disease

Vitamin B12 and folate deficiency

Toxins and Drugs

Metals

Organic compounds

Carbon monoxide

Drugs

Brain TumoursTrauma

Open and closed head injuries

Punch-drunk (boxers) syndrome

Heat stroke

Subdural haematoma

Infections

Brain abscess

Bacterial meningitis

Fungal meningitis

Encephalitis

Subacute sclerosing

panencephalitis

Progressive multifocal

leukoencephalopathy

Jakob-Creutzfeldt disease

Kuru

Behcet's syndrome

Lues

Other Diseases

Multiple sclerosis

Muscular dystrophy

Whipple's disease

Concentration-camp syndrome

Kuf's disease

Familial calcification of  
the basal ganglia<sup>a</sup> Modified from Haase (1977)



(Blocq and Marinesco, 1892), the intracellular accumulations of fibrous material had not and became known as Alzheimer's neurofibrillary changes or, more recently, neurofibrillary tangles.

Since Alzheimer first described the neuropathological changes of senile plaques and neurofibrillary tangles in a 55 year-old patient the term Alzheimer's disease has been used primarily to describe the disorder that occurs in the presenile (under 65 years-old) age group. However, identical neuropathological features appear associated with dementia in the over-65 age group and since the qualitative neuropathological and clinical changes that occur in the two age groups appear essentially identical (Terry, 1976; Constantindis, 1978; Tomlinson, 1980) - although the younger patients may show greater quantitative changes (Corsellis, 1976; Mann et al, 1984). Consequently, it is now generally accepted that there is no major distinction between pre-senile and senile forms and that the term dementia of the Alzheimer-type can be used for both age groups. In the work presented here the term senile dementia of Alzheimer-type (SDAT) is used merely to indicate that the subjects of this study were over 65 years old and does not imply any clinical or neuropathological difference between pre-senile and senile dementia.

### 3. Clinical symptoms of SDAT

The cognitive dysfunction associated with SDAT is chronic and progressive, with accompanying deterioration of personality. Although the symptoms can be roughly divided into three stages, the gradual progression of the syndrome means that distinctions between the stages are not clearcut (Schneck et al, 1982).

Stage one; the "forgetfulness stage". The cognitive deficits are not very pronounced. The patient becomes forgetful and shows difficulty remembering names and appointments or where objects are placed. The range of interest of the patient diminishes and the emotions become blunted. Attempts to persuade the patient to undertake quite ordinary tasks may evoke unexpected anger. Depression or anxiety often accompanies the early stages

Stage two; the "confusional stage". At this stage a definite cognitive impairment, which is most severe with respect to memory of recent events, becomes manifest. Orientation and concentration become affected and, whilst the vocabulary is largely spared, the individual may



have difficulty recalling appropriate words.

Stage three; the "dementia stage". The patient becomes isolated from visual and auditory contact with the environment and emotional responses are severely reduced. There may be transient episodes in which the patient is found talking to himself. Severe disorientation becomes manifest and may result, for example, in the patient confusing a spouse with a parent. The vocabulary becomes impoverished, with phrases and tenses simplified and processes of metaphor and analogy reduced. Somatic and neurological abnormalities may appear including incontinence and abnormal reflexes. In addition delusions, hallucinations and severe agitation may become manifest.

#### 4. Epidemiology of SDAT

Precise figures for the prevalence of SDAT are not available although estimates have been made of the prevalence of "organic" dementias, which include SDAT. Thus, approximately 6-15% of over 65 year olds have been estimated to suffer from organic dementia (Kay et al, 1970; Terry and Katzman, 1983), of which approximately two-thirds have been estimated to be affected by a mild form in which the individual shows signs of intellectual impairment but is still able to carry out the activities of daily living. The remaining one-third suffer from severe dementia with a characteristic disorganization of personality and inability to carry out the normal tasks of daily living (Terry and Katzman, 1983). Furthermore, the incidence of dementia in over 65 year olds is age related since it has been estimated that the symptoms occur in 2.4% of the 65-69 age group compared to 22% of those aged 80 and above (Kay et al, 1964, 1970).

Assuming that there is a prevalence of organic dementias in the elderly of 15% and that 50% of organic dementia cases are due to Alzheimer's disease (Tomlinson et al, 1970; Jellinger, 1976) it becomes apparent that approximately 7.5% of over 65 year-olds are affected by SDAT, which in the United States (for which figures are most readily available) represents approximately 500,000 and 1,000,000 cases of advanced and moderate SDAT respectively, or a total prevalence of greater than 8 per 1000 of the whole population (Katzman, 1976; Terry, 1976). In addition, it has been estimated that in the United States, 100,000 deaths occur each year related to SDAT (Katzman, 1976; Terry, 1976; Schneck,

1982) and it therefore ranks as the fourth or fifth most common cause of death in the United States (Katzman, 1976). Consequently, as the proportion of elderly continues to be the fastest growing age-group in the Western world, so both the emotional and financial burden on society caused by SDAT are increasing to epidemic proportions (Plum, 1979).

### 5. The diagnosis of dementia and SDAT

The present section discusses the problems associated with the diagnosis of SDAT and is divided into two sections. Firstly, the problems of differentiating SDAT from other disorders that present with similar symptoms are described and secondly, the use of the electroencephalogram and computerised brain scanning techniques in the diagnosis of SDAT are discussed.

#### a) Differentiation of SDAT from disorders with similar symptoms

The diagnosis of SDAT is primarily a process of eliminating of all other possible causes of the dementia. A diagrammatic scheme of diagnostic decisions is shown in figure 1 (modified from Gurland and Toner, 1983).

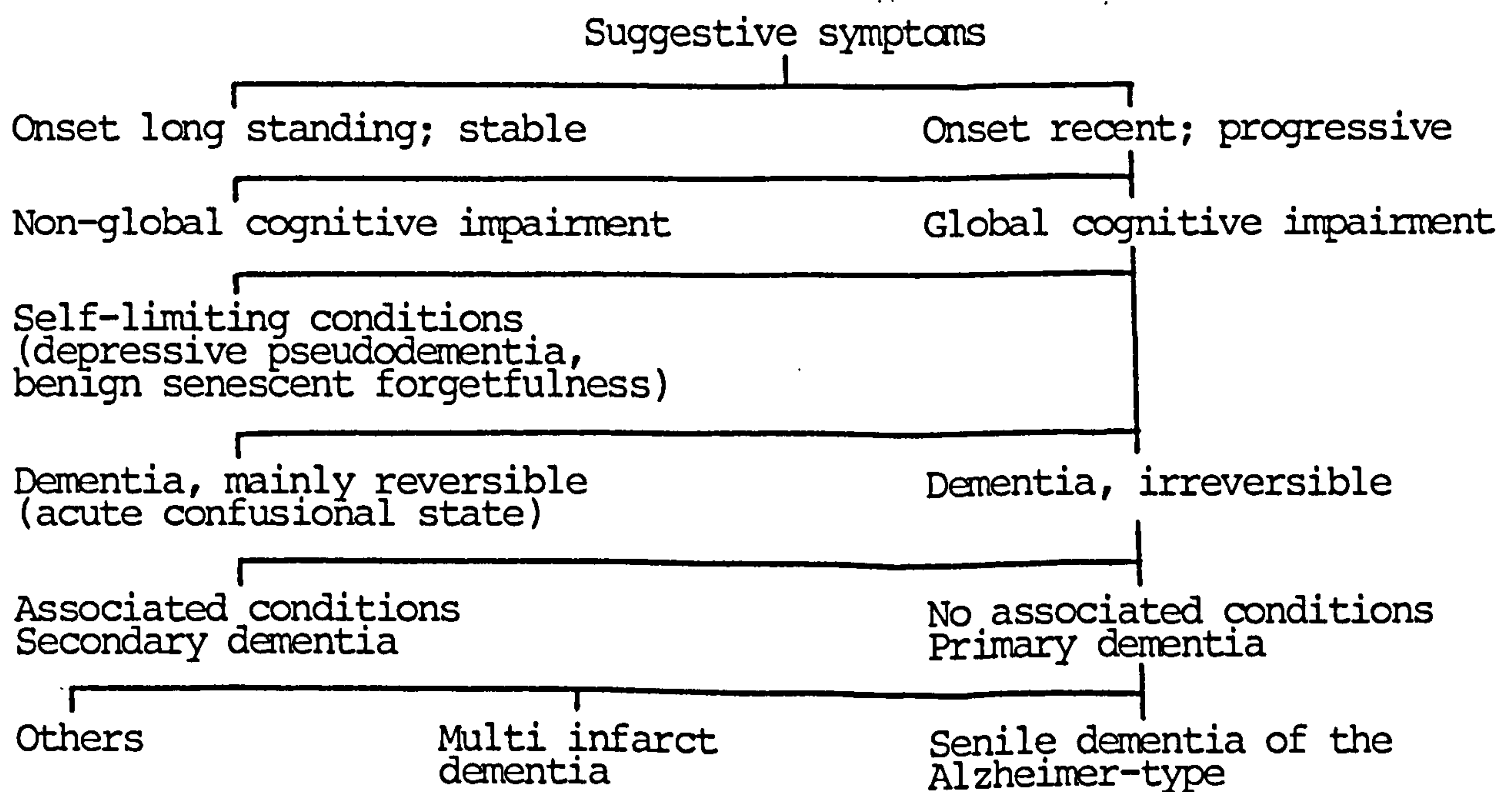


Figure 1. Schematic representation of the decisions to be made for accurate diagnosis of SDAT. (Modified from Gurland and Toner, 1983).



The decisions to be made are (see also Gurland and Toner, 1983); First, are the suggestive symptoms long standing and stable or recent and progressive?

Prolonged observation or a careful history from an informant are very useful and since the majority of dementing processes are progressive, it is unlikely that any long-standing symptoms are due to a dementing process.

Second, is the impairment global or non-global?

Dementia characteristically involves all cognitive functions, though not necessarily in a uniform manner. Global intellectual impairment is most readily assessed using tests of orientation and memory or using a history of the patients everyday functions.

Third, is the cognitive impairment due to self limiting conditions?

The next step in the diagnosis is to eliminate self limiting conditions, the two most important categories of which are benign senescent forgetfulness and depressive pseudodementia.

Benign senescent forgetfulness was described by Kral (1962) as being a separate entity to the malignant amnesic syndrome. Upon formal testing the person suffering from benign senescent forgetfulness shows none of the range of errors expected of dementia.

Depressive pseudodementia describes a syndrome which, to a varying degree, resembles dementia, but is actually due to a disordered mood of depression (Kiloh, 1961). The simplest reason for the overlap between the symptoms of depression and dementia is that the depressed patient complains about memory disturbances which in the normal elderly person would be classed as normal lapses of memory. In addition, the depressed patient is not only more sensitive to memory lapses but is also likely to have an increased tendency for such lapses due to a preoccupation with brooding thoughts.

Fourth, are the symptoms due to the acute confusional state?

Having eliminated depressive pseudodementia and benign senescent forgetfulness as causes of the symptoms, the next step is to try and identify whether the symptoms are due to the acute confusional state - which constitutes 10-30% of the total diagnoses of dementia (Cumming, 1983).

The acute confusional states are usually due to exogenous factors (i.e. arising outside the brain) such as toxic conditions, nutritional and endocrine disorders, infections, and cerebral disease and are treatable and in many cases reversible (for detailed discussion see



Cummings, 1983).

Fifth, is the dementia primary or secondary?

Having finally reached a diagnosis of progressive irreversible dementia the next step is to establish whether the dementia is primary, i.e. is the dementia the major clinical symptom or is it secondary to other major clinical symptoms? The primary dementias, which constitute the major cause of all dementia, mainly consist of cases showing Alzheimer-type pathology (SDAT; 50%) or arteriosclerotic changes (multi-infarct dementia; 20%) or both (15%; Tomlinson et al, 1970; Tomlinson, 1980). The diagnosis of multi-infarct dementia is fairly easily made since the step-wise onset and the occurrence of signs of focal brain disease have allowed the construction of simple scoring criteria for its diagnosis (Hachinski et al, 1975), which have been subsequently validated pathologically (Rosen et al, 1980).

SDAT is therefore diagnosed on the basis of the elimination of all other possible causes of dementia. Misdiagnoses of dementia occur to varying degrees (see Gurland and Toner, 1983) due mainly to pseudodementia, and focal deficits of higher neurological disorders which may be mistaken for global cognitive impairment. None of the various psychometric or neurological testing procedures employed by different investigators are sensitive or selective enough to differentially diagnose early SDAT from the various disorders presenting as dementia, and it is only as SDAT becomes more severe that an accurate diagnosis may be made. Unfortunately it is in these latter stages, with gross involvement of many higher functions, that identification of the core disabilities, which would presumably be the most responsive to drug therapies is most difficult.

b) Use of the electroencephalogram and brain scans in diagnosis of SDAT

The electroencephalogram (EEG) represents the summed electrical potential changes from large areas of the brain and as such would seem to present a method of evaluating the changes that occur throughout the cortex in SDAT. In dementia it has been shown that the high frequency bursts of electrical activity become less frequent and are replaced by slower rhythms (reviewed by Pedley and Miller, 1983). However, problems occur in quantification and interpretation of data and deciding whether changes are normal consequences of old age or indications of pathological dysfunction (Pedley and Miller, 1983). Similarly, recent measurements of the latency of the evoked P<sub>300</sub> potential (i.e. the potential detected



with a latency of 300msec after application of a stimulus) also suggest that this parameter is of limited use as an index of dementia, (Pfefferbaum et al, 1982).

The use of computerised tomographic (CT) scanning to construct X-ray images of the brain in SDAT has so far been restricted to the examination of gross pathological changes such as CSF distributions and dilation of the cortical sulci. However, observations have, to date, been inconclusive (for review see de Leon and George, 1983) and since many of the macroscopic changes that occur in dementia also occur in normal aging and even in severe cases of dementia, gross structural changes may be absent (Tomlinson et al, 1970) the usefulness of CT scans in the diagnosis of dementia remains to be established (de Leon and George, 1983).

The advent of positron emission tomography (PET) allows, in contrast to the structural changes detected in CT scans, regional changes in brain function to be measured (for example the use of  $^{18}\text{F}$ -2-deoxy-2-fluoro-D-glucose as a glucose analogue gives quantitative measurements of regional brain metabolism). Using this technique, although there is controversy over whether or not age-related changes occur, there does appear to be agreement that SDAT associated decreases do occur (Ferris et al, 1983; Friedland et al, 1983) and furthermore cases of SDAT and multi-infarct dementia have been discriminated (Benson et al, 1983).

In addition to EEG and tomographic techniques, numerous other studies have been carried out in the search for an SDAT-specific biochemical marker in both the blood and cerebrospinal fluid. The results obtained in these studies are discussed in more detail elsewhere (chapter 4, section I).

## 6. Neuropathological features of SDAT

In the present section, both the macroscopic and microscopic changes associated with SDAT are described. Since the characteristic neuropathological changes associated with SDAT are microscopic rather than macroscopic, particular attention is paid to the former type of changes. Thus, the four types of microscopic lesion found in the SDAT brain, namely the granulovacuolar degeneration and Hirano body and particularly the senile plaque and neurofibrillary tangle, will be described.



### a) Macroscopic changes

It is often considered that "the brain in either Alzheimer disease or senile dementia is shrunken in size and weight, with lessened cortex and white matter, and consequently enlarged ventricles" (Terry, 1976). There have, however, been surprisingly few studies concentrating on the gross rather than the microscopic pathological features of the disorder. When total brain weight has been reported there is a conflict for whilst several groups have reported no change in brain weight compared to age-matched normals (Tomlinson et al, 1970; Bowen et al, 1976; Mann et al, 1980, 1982; Terry 1981; DeKosky and Bass, 1982; Rylett et al, 1983). On the other hand, slight (approximately 10%) but significant reductions in brain weight have been reported to occur in SDAT (Terry et al, 1981; Rossor et al, 1982b). Neither of these latter reports studied the brains of patients from the presenile age group in which the brain is indeed much smaller (Terry and Davies, 1980) with a reported loss of weight from the usual level of 1200 to 1450g to often less than 1000g (Corsellis, 1976).

Furthermore, neither were the subjects all less than 80 years old, at which age it has been reported that global atrophy occurs, whereas in patients over 80 years of age only the temporal lobe is atrophic (Hubbard and Anderson, 1981). The difference between the groups that did and did not observe changes in brain weight may be related to the severity of cases examined since Bird et al (1983) have observed the brains of SDAT subjects characterised by more severe microscopic changes are significantly lighter than less affected brains. In addition, there does not appear to be a gross loss of cortical grey matter since cortical thickness has been reported to be unchanged in SDAT brains compared to the normal (Terry et al, 1981; DeKosky and Bass, 1982).

### b) Microscopic changes

The main neuropathological features of SDAT are the presence of neocortical neurofibrillary tangles (tangles) and senile plaques. Although Alzheimer first described these lesions in the cortex (Alzheimer, 1907), they also occur, although far less frequently, in other regions of the brain. Thus, plaques may occasionally be found in the mamillary bodies and more rarely in the basal ganglia, hypothalamus, upper brainstem and the cerebellum (Tomlinson, 1982). Similarly, tangles also occur outside the cortex and have been reported in many regions of the brain including the raphe nucleus (Ishii, 1966), substantia nigra and the locus coeruleus and may be found less frequently in the lower



brainstem, cerebellum or spinal cord (Hirano and Zimmerman, 1962; Tomlinson, 1982).

Senile plaques and neurofibrillary tangles are not, however, found exclusively in SDAT since they are frequently present not only in normal elderly brains (particularly in archicortical regions such as the hippocampus and amygdala) but also in other diseases of the central nervous system (see below). In general, however, it is the number of these lesions that distinguishes cases of SDAT from normal elderly subjects. Hence, it is not the mere presence of these lesions which distinguishes SDAT from the non-demented cases, rather it is the presence of much larger numbers of plaques and tangles in SDAT that is the distinguishing feature (Tomlinson et al, 1968, 1970; Tomlinson, 1982). Furthermore, although cortical plaque formation in non-demented elderly persons may reach similar proportions to the numbers observed in SDAT cases (Tomlinson and Henderson, 1976), neocortical tangles are usually absent from such brains and are only found in large numbers in patients who are demented (Tomlinson et al, 1970).

In addition to their occurrence in elderly normal as well as SDAT cases plaques and tangles also occur in other diseases of the central nervous system (Tomlinson 1982). Thus, senile plaques are also found in a minority of cases dying from the "slow virus" dementing disorders of Kuru and Jakob-Creutzfeldt disease, and are also present in large numbers in the majority of cases of Down's syndrome surviving to middle life. Similarly, neurofibrillary tangles are also found in adult cases of Down's syndrome and have also been described in dementia pugilistica ("boxer's dementia") and the Parkinson-dementia and amyotrophic lateral sclerosis complexes of Guam, as well as various other disorders (Hirano and Zimmerman, 1962; Tomlinson, 1982).

There now appears to be general agreement on the structure of the plaques and tangles found in SDAT and the following description is derived from the recent review by Tomlinson (1982). Plaques vary from 15 to 200µm in diameter and consist of enlarged terminals (often called neurites) and nerve processes surrounding a central core of amyloid fibrils with associated microglia and astrocytic processes present. The enlarged neurites are distended with lysosomes, abnormal mitochondria and paired helical filaments, and appear to be mainly of presynaptic origin. At the ultrastructural level, the smallest (earliest) plaques have three or four distended neurites (without amyloid) which subsequently develop into the mature plaque which consists of a mass of central amyloid with



few surrounding neurites. Histochemically the plaques, which appear to be identical in senile and pre-senile forms subjects (Friede, 1965), are associated with increased hydrolytic activity (including acetylcholinesterase and butyrylcholinesterase) in addition to which nucleotide phosphatase and oxidative activities are often increased (Friede, 1965; Corsellis, 1976).

In contrast to the senile plaques, which are extracellular abnormalities, neurofibrillary tangles are intracellular structures which may occupy most of the cytoplasm of the neuronal cell body. They consist of large clusters of paired helical filaments, which are coiled around each other and are 20nm at their widest point which reduces to 10nm at intervals of 80 nm. These fibres are very insoluble (Selkoe et al, 1982) and neurons containing tangles are associated with increased deposits of aluminium in the neuronal nuclei (Perl and Brody, 1980).

The numbers of plaques and tangles have both been found to correlate with the clinical severity of the dementia. The correlation of plaques with severity of dementia has been reported by several groups (Corsellis, 1962; Blessed et al, 1968; Farmer et al, 1976; Wilcock and Esiri, 1982), whilst the number of tangles also correlates with dementia (Farmer et al, 1976; Wilcock and Esiri, 1982). However, whilst it has been suggested that the number of tangles is a better correlate of dementia than the number of plaques (Wilcock and Esiri, 1982; Wilcock et al, 1982), Terry et al (1982) have described a group of older demented patients with abundant cortical plaques but an absence of neocortical tangles.

Simchowicz (1911) described an additional lesion, granulovacuolar degeneration, in the pyramidal cells of the hippocampus in senile dementia. The cytoplasm of the affected cell contains one or more clear vacuoles (3 to 5µm in diameter) at the centre of which is an haemotoxyphilic and argyrophilic granule about 1µm in diameter (Corsellis, 1976). In contrast to the plaques and tangles, which are found in large numbers in both the hippocampus and neocortex in SDAT, granulovacuolar degenerations are almost exclusively confined to the hippocampus. Whilst these lesions are also found in normal elderly cases, it is - as with the plaques and tangles - their much greater abundance in SDAT that distinguishes the pathological brain from the normal (Tomlinson, 1982). In addition to SDAT, granulovacuolar changes are also observed in other pathological situations such as adult Down's syndrome, Pick's disease and the amyotrophic lateral sclerosis and Parkinson-dementia complexes of Guam (Corsellis, 1976).

In addition to plaques, tangles and granulovacuolar degenerations, a fourth type of lesion, the Hirano body, is also found in SDAT. Hirano bodies are eosinophilic oval or rod-like bodies that are 8-15 $\mu$ m across and about 30 $\mu$ m long. Like the granulovacuolar degenerations, they are found mainly in the pyramidal cell layers of the hippocampus, although they may very occasionally be found in other areas of the cortex (Tomlinson, 1980).

Using Golgi staining techniques, dendritic abnormalities have been shown to occur both in normal aging and to a greater extent in SDAT. Thus, a loss from the cortical pyramidal cells of both dendritic arbor and dendritic spines has been reported to occur in SDAT in excess of the loss observed as a consequence of normal aging (Mehraein et al, 1975; Schiebel and Scheibel, 1975; Scheibel 1978). In addition, whilst an increase rather than a decrease in dendritic arbor as a consequence of normal aging was observed in pyramidal cells of the hippocampus by Buell and Coleman (1979), they also observed that the dendritic arbor was less extensive in cases of SDAT.

Throughout life neocortical neurons appear to be lost as a result of normal aging and cell loss over and above that observed in normal aging has been reported in SDAT (see Tomlinson, 1982). More specifically, there appears to be a loss of the larger neocortical neurons (Terry et al, 1981). Neuronal loss has also been observed in non-neocortical regions such as the hippocampus (Ball, 1977), the cholinergic nucleus basalis of Meynert (section 7.a.i), the noradrenergic locus coeruleus (Forno, 1978; Tomlinson et al, 1981; Bondareff et al, 1982) and the olfactory bulbs (Esiri and Wilcock, 1984).

## 7. Neurochemistry of SDAT

In the 1960's work was carried out that was to establish that in Parkinson's disease, the neurons that project from the substantia nigra to the caudate nucleus and putamen, and which had previously been reported by neuropathologists to be degenerated, were deficient in the neurotransmitter dopamine (see Hornykiewicz, 1973). The subsequent treatment of the disease by the administration of the dopamine precursor L-Dopa (dihydroxyphenylalanine) in order to increase the levels of the neurotransmitter has proved - at least in the majority of cases - to be of great therapeutic benefit. This classical example of neurochemical



research into Parkinson's disease demonstrates that not only may a disease of the central nervous system (CNS) be associated with the loss of a discrete neuronal population characterised by their use of a common neurotransmitter, but also that the transmitter deficiency may be treatable.

In SDAT, the majority of the early neurochemical studies concentrated on the levels of various general metabolic enzymes along with the levels of nucleic acids and miscellaneous other enzymes, proteins, lipids and carbohydrates (for review see Terry and Davies, 1980). However, more recent neurochemical research has focussed on the distribution and activities of neurochemical "markers" associated with different neurotransmitter systems in the hope of identifying neuronal populations that are selectively involved in SDAT and therefore analogous to the dopaminergic neurons in Parkinson's disease.

The present section will therefore review the data that exists for the involvement of different neurotransmitter systems in SDAT in the CNS (see chapter 4, section I for discussion of neurochemical markers in the cerebrospinal fluid). For convenience the present section has been divided into two main parts based upon whether the neurons contain one of the classical neurotransmitter substances (i.e. acetylcholine, noradrenaline, serotonin or dopamine) or alternatively contain amino acids or neuropeptides in relation to neurotransmission.

The evidence discussed suggests that neurons containing acetylcholine, serotonin, noradrenaline and somatostatin may all, to varying degrees, be involved in SDAT.

a) Neurons containing acetylcholine, noradrenaline, serotonin and dopamine in SDAT

i) Acetylcholine

To date, the central nervous neurotransmitter system that appears to be most extensively involved in SDAT is the cholinergic system - as judged by the levels and activities of markers (section II.3.a) of central cholinergic neurons (table 2). Thus, although a deficit in acetylcholinesterase (AChE) was initially reported in the neocortex of patients showing Alzheimer-type neuropathology in the mid-1960's (Pope et al, 1964, 1965), the implications of this finding were not fully appreciated, presumably due to a lack of understanding of the central cholinergic system and doubts as to the validity of studies carried out on postmortem material. These initial results were, however,



TABLE 2  
Involvement of the central cholinergic system in SDAT

I) Biochemistry of the cholinergic system in SDAT<sup>a</sup>

Observation	References	
a) Decreased cortical AChE and ChAT activity	Pope et al, 1964, 1965; * Davies & Maloney, 1976; * Spillane et al, 1977; * Bowen et al, 1979	Bowen et al 1976 Perry et al, 1977b White et al, 1977
b) Decreased AChE and ChAT in the nucleus of Meynert	Perry et al, 1982b; Bird et al, 1983;	Rossor et al, 1982c Candy et al, 1983
c) Decreased acetylcholine synthesis	* Sims et al, 1980, 1981, 1983a * Bowen et al, 1983	
d) Decreased high-affinity choline uptake	* Sims et al, 1983a;	Rylett et al, 1983
e) Decreased levels of acetylcholine	Richter et al, 1980	
f) Muscarinic receptor binding normal	Perry et al, 1977b, 1978b Davies & Verth, 1978; b Caulfield et al, 1982 Lang & Henke, 1983	White et al, 1977 Bowen et al, 1979 Norberg et al, 1982; Cross et al, 1984
reduced in certain cases	Reisine et al, 1978; Rinne et al, 1984	Wood et al, 1983
g) Nicotinic receptors normal	Norberg et al, 1982;	Lang & Henke, 1983
reduced	Davies & Feisullin, 1981	

II) Histochemistry and immunocytochemistry

Observation	References	
a) AChE (and BChE) associated with senile plaques	Friede, 1965; Struble et al, 1982	Perry et al, 1980
b) AChE-staining fibres lost from the cortex	Perry et al, 1980, 1983a; Candy et al, 1983	
c) AChE and ChAT staining lost from or changed the nucleus of Meynert	Candy et al, 1983; Parent et al, 1984	Pearson et al, 1983c McGeer et al, 1984

III) Neuronal loss or degenerative changes in the nucleus of Meynert

Averback, 1981	Whitehouse et al, 1981, 1982	Perry et al, 1982b, 1983a
Arendt et al, 1983	Candy et al, 1983	Tagliavini & Pilleri, 1983
Wilcock et al, 1983	Mann et al, 1984	

<sup>a</sup> See table 24 for cholinergic-related parameters in the cerebrospinal fluid

<sup>b</sup> Includes different receptor subtypes

\* Biopsy samples

substantiated by the simultaneous discovery by three groups of a deficit in the acetylcholine (ACh)-synthesising enzyme choline acetyltransferase (ChAT; Bowen et al, 1976; Davies and Maloney, 1976; Perry et al, 1977b).

Subsequently, numerous reports have confirmed that the activity of both AChE and ChAT are reduced in postmortem samples of cortex obtained from patients suffering from SDAT (reviewed by Perry and Perry, 1980; Bartus et al, 1982; Marchbanks, 1982; Coyle et al, 1983). Furthermore, the observations that cholinergic-related activities are reduced in postmortem material have been extended to biopsy samples (Spillane et al, 1977; Bowen et al, 1979).

Not only are the levels of the degradative and synthetic enzymes reduced in SDAT, but also the levels of ACh itself have been found to be significantly reduced in postmortem SDAT temporal cortex (Richter et al, 1980) - although it has been suggested that postmortem levels represent only 5% of the levels present before death (Marchbanks, 1982). Furthermore, in recent metabolic studies using biopsy neocortical samples, a reduced capacity for ACh synthesis (which was not due to a general reduction in metabolic activity since carbon dioxide production from glucose actually increased) has been demonstrated (Sims et al, 1980, 1981, 1983a,b; Bowen et al, 1983). Using similar biopsy neocortical samples it has also been shown that the activity of the presynaptic high-affinity choline uptake (HACUP) system is reduced in SDAT (Sims et al, 1983a). Reduced activity of the HACUP system has also been demonstrated in synaptosomes prepared from postmortem samples of both hippocampus and frontal cortex (Rylett et al, 1983).

The loss of various cholinergic markers associated with the presynaptic nerve terminal (i.e. ACh, AChE, ChAT and HACUP) from the cortex all suggest that there is a degeneration of these structures in SDAT and in agreement with this is the observation that AChE-staining fibres are lost from the cortex in SDAT (Perry et al, 1980, 1983a; Candy et al, 1983). Interestingly, histochemical AChE-staining has been demonstrated in the region of the senile plaque and suggests that AChE may be released from degenerating cholinergic neurites associated with these structures (Friede, 1965; Perry et al, 1980; Struble et al, 1982).

In marked contrast to the loss of cholinergic related activities associated with the presynaptic nerve terminal, the postsynaptic cholinergic receptor binding appears to be largely unaltered. Hence, the levels of cholinergic muscarinic receptor binding, including different receptor subtypes (Caulfield et al, 1982) appear to be, on the whole,



normal (table 2) although reduced levels have been observed in the limbic areas by Reisine et al (1977) and Rinne et al (1984) whilst Wood et al (1983) identified a sub-population of SDAT cases with reduced neocortical muscarinic binding. Nicotinic binding also appears normal as judged by the binding of tubocurarine (Norberg et al, 1982). Using  $\alpha$ -bungarotoxin binding, which may not, however, be specific for nicotinic receptors (see Fibiger, 1982), both normal (Norberg et al, 1982; Lang and Henke, 1983) and reduced (Davies and Feisullin, 1981) levels of putative nicotinic receptor binding have been reported in SDAT. Interestingly, after lesioning of the cholinergic septo-hippocampal pathway in the rat, an analogous situation to that observed in the SDAT cortex occurs in the hippocampus (i.e. loss of presynaptic markers such as AChE, ChAT and HACUP but normal levels of postsynaptic receptor binding sites; see chapter 3, section I).

In agreement with the probable loss of presynaptic nerve terminals in SDAT, the nucleus of Meynert (which is thought to be the major source of afferent cortical cholinergic activity; section II.3.c), also appears to be involved in the pathology of SDAT (table 2). Thus, not only are neuronal cell bodies lost or shrunken in this region, but also both AChE and ChAT activities are reduced, although no alterations in the levels of ChAT activity were observed by Henke and Lang (1983).

Whilst the physiological function of BChE remains uncertain (section II.7.d), levels of this enzyme have been observed to be increased in the water-soluble fraction of SDAT cortex (Op den Velde and Stam, 1976) as well as in whole cortical homogenates (Perry et al, 1978a,b). In addition, the BChE activity in the hippocampus has been found to correlate with the degree of granulovacuolar degeneration (Perry and Perry, 1980). Histochemically, BChE-staining has been observed associated with the senile plaques (Friede, 1965). However, the pathological significance of these results remains to be determined.

The importance of the cholinergic system in the underlying pathological processes of SDAT is suggested not only by the correlation of the degree of cholinergic deficit with both the clinical and neuropathological severity of the disorder (Bowen et al, 1976; Perry et al, 1978b, 1981a; Wilcock et al, 1982; Mountjoy et al, 1984), but also by the observation that the administration of drugs aimed at the central cholinergic system produce a deficit in memory similar to that observed in SDAT (section 8).



## ii) Noradrenaline

The noradrenergic system is implicated in SDAT since there is a loss of neurons in the locus coeruleus which is the source of the noradrenergic afferent input to the cortex (table 3). Furthermore, the locus coeruleus also appears to be susceptible to neurofibrillary tangles in Alzheimer-type dementia (Hirano and Zimmerman, 1962).

Biochemically, although there appears to be agreement that levels of the transmitter itself, noradrenaline, are reduced in SDAT (table 3), the activity of the noradrenergic marker enzyme dopamine- $\beta$ -hydroxylase (DBH) has been reported to be reduced (Cross et al, 1981; Perry et al, 1981b,c), although in a preliminary study, unaltered levels of DBH were observed in the cortex of SDAT subjects by Davies and Maloney (1976). Furthermore, the number of DBH-positive cells in the locus coeruleus is reduced in the brains of SDAT subjects (Iversen et al, 1983). Interestingly, DBH levels have also been reported to be decreased in both the CSF and serum of SDAT subjects (Miyata et al, 1984). The data concerning the levels of the noradrenaline metabolite, 3-methoxy-4-hydroxyphenylglycol (MHPG) in the cortex are controversial for whilst the Swedish group have reported elevated levels (Gottfries et al, 1983), significant reductions in these areas have also been reported (Perry et al, 1981b; Cross et al, 1983). However, normal levels of MHPG have been reported in the CSF (chapter 4, section I)

In addition to the general agreement that the levels of noradrenaline itself are reduced in SDAT, the reuptake of noradrenaline into the presynaptic nerve terminals is reduced in biopsy samples of SDAT neocortex (Benton et al, 1982). Furthermore, a loss of noradrenergic axons from the cortex has been reported in SDAT (Berger et al, 1976) and those that remain appear abnormal (Berger and Alvarez, 1983). Interestingly, and analogous to the situation concerning the cholinergic system, despite the loss of presynaptic markers there does not appear to be a loss of the postsynaptic noradrenergic  $\alpha_1$ ,  $\alpha_2$  or  $\beta$ -receptors (Bowen et al, 1979, 1983; Cross et al, 1984).

Whilst these data suggest that there may be a degeneration of subcortical afferents from the locus coeruleus analogous to the degeneration of cholinergic afferents from the nucleus of Meynert, the noradrenergic locus coeruleus cell loss does not, however, parallel the cholinergic involvement. Thus, significant reductions in ChAT have been reported to occur irrespective of whether or not there was substantial cell loss in the locus coeruleus (Perry et al, 1981b). Furthermore,

TABLE 3  
Involvement of the noradrenergic system in SDAT

I) Biochemistry of the noradrenergic system in SDAT<sup>a</sup>

Observation	References	Areas studied
a) DBH activity		
reduced	Cross et al, 1981	frontal & temporal cortex, hippocampus
	Perry et al, 1981b,c	frontal & temporal cortex
normal	Davies & Maloney, 1976	not stated
b) Reduced NA-uptake	* Benton et al, 1982	temporal cortex
c) Reduced NA	Adolfsson et al, 1979	frontal cortex & putamen
	Gottfries, 1980	caudate nucleus
	Yates et al, 1981	hypothalamus
	Mann et al, 1982	caudate nucleus, hypothalamus
	Gottfries et al, 1983	cingulate cortex, hippocampus, caudate nucleus
	Iversen et al, 1983	temporal cortex
	Yates et al, 1983a,b	hypothalamus, mamillary body
	Rossor et al, 1984	temporal and cingulate cortex, hippocampus
d) MHPG		
reduced	Cross et al, 1983	frontal, temporal & occipital cortex, hippocampus
increased	Gottfries, 1980	caudate nucleus
	Gottfries et al, 1983	cingulate cortex, hippocampus, caudate nucleus
e) Normal $\beta$ -receptors	Bowen et al, 1979	temporal cortex
	Bowen et al, 1983	frontal cortex
	Cross et al, 1984	occipital cortex, hippocampus
f) Normal $\alpha$ 1 & $\alpha$ 2 receptors	Cross et al, 1984	occipital cortex, hippocampus

II) Histochemical and immunohistochemical observations

Noradrenergic fibres lost or abnormal in the cortex	Berger et al, 1976; Berger & Alvarez, 1983
Loss of DBH-positive cells in the locus coeruleus	Iversen et al, 1983

III) Neuronal loss in the locus coeruleus

Forno, 1978	Mann et al, 1980, 1982, 1984	Perry et al, 1981b,c
Tomlinson et al, 1981	Bondareff et al, 1982	Iversen et al, 1983

<sup>a</sup> See table 24 for noradrenergic-related parameters in the cerebrospinal fluid

\* Biopsy samples



neither the reductions in levels of MHPG, DBH nor locus coeruleus cell counts were found to correlate with the severity of dementia (Perry et al, 1981c; Cross et al, 1983) and it would therefore appear that the involvement of the noradrenergic system in SDAT is a secondary phenomenon.

### iii) Serotonin

Neuropathologically the serotonergic system has been implicated in SDAT by the presence of neurofibrillary tangles in the serotonergic Raphe nucleus and dorsal tegmental nucleus (Ishii, 1966; Mann and Yates, 1983).

Biochemically (see table 4), although normal levels of both serotonin (5-hydroxytryptamine) and the serotonin metabolite 5-hydroxy-indoleacetic acid (5-HIAA) have been reported (Adolfsson et al, 1979; Reynolds et al, 1984) other reports suggest that levels of both these chemicals are decreased in postmortem SDAT brain samples (table 4) as is the level of serotonin plus 5-HIAA (Bowen et al, 1983). In the CSF, the levels of 5-HIAA have been reported to be both normal and reduced (chapter 4, section I). The reduced serotonergic activity in SDAT suggested by the reports of reduced levels of serotonin and 5-HIAA in the postmortem brain, have been substantiated by the observation that serotonin uptake is reduced in biopsy samples of SDAT neocortex (Benton et al, 1982; Bowen et al, 1983).

In addition to the loss of presynaptic markers (and in contrast to both the cholinergic and noradrenergic systems) there also appears to be a loss of cortical postsynaptic serotonin receptors (Bowen et al, 1979, 1983) and more specifically the  $S_1$  and  $S_2$  receptors (Cross et al, 1984; Reynolds et al, 1984).

Although a serotonergic-cholinergic interaction has been suggested to occur within the central nervous system (Butcher and Woolf, 1982) and, more specifically, has been demonstrated in the rat cortex (Robinson, 1984), the possibility that the serotonergic loss of activity in SDAT is directly associated with the loss of cholinergic activity does, however, seem unlikely. Thus, whereas reductions in ChAT were observed in all cases studied by Bowen et al (1983), the changes in postsynaptic serotonergic receptors occurred only in certain Alzheimer-type specimens indicating that the serotonin receptor alterations may be secondary to the cholinergic changes. In addition, when the cell bodies of the basal forebrain region of the rat corresponding to the primate nucleus of Meynert are destroyed by kainic acid injections, the marked loss of

TABLE 4  
Involvement of the serotonergic system in SDAT

Biochemistry of the serotonergic system in SDAT<sup>a</sup>

Observation	References	Areas studied
a) Serotonin		
reduced	Gottfries, 1980	caudate nucleus
	Gottfries et al, 1983	cingulate cortex, hippocampus, caudate, hypothalamus
normal	Adolfsson et al, 1979	frontal & cingulate cortex, hippocampus
	Reynolds et al, 1984	frontal cortex
b) 5-HIAA		
reduced	Bowen et al, 1979	whole temporal lobe
	Adolfsson et al, 1979	cingulate cortex, hippocampus
	Cross et al, 1983	frontal & temporal cortex, hippocampus
normal	Gottfries, 1980	caudate nucleus
	Gottfries et al, 1983	cingulate cortex, hippocampus, caudate, hypothalamus
	Reynolds et al, 1984	frontal cortex
c) reduced serotonin uptake	* Benton et al, 1982	temporal cortex
d) reduced receptor binding	Bowen et al, 1979	whole temporal lobe
	Reynolds et al, 1984	frontal cortex
	Cross et al, 1984	frontal & temporal cortex, hippocampus

<sup>a</sup> See table 24 for serotonergic-related parameters in the cerebrospinal fluid

\* Biopsy samples



cortical cholinergic activity is not accompanied by any alteration in cortical serotonergic activity as judged by both serotonin levels (Johnston et al, 1979) and serotonin uptake (Bowen et al, 1983). Indeed it has been suggested that changes in the serotonergic neuronal system may more closely parallel changes in the noradrenergic rather than the cholinergic system (Reynolds et al, 1984).

#### iv) Dopamine

The involvement of the dopaminergic system in SDAT is, at the moment, controversial (table 5). Thus, whilst reduced levels of dopamine and the dopamine metabolite homovanillic acid (HVA) have been reported in autopsy brain tissue by the Swedish group (Gottfries et al 1969b; Adolfsson et al, 1979; Gottfries, 1980; Gottfries et al, 1983), results obtained by other groups suggest that in brain tissue of SDAT subjects, levels of both dopamine and HVA are normal (Yates et al, 1979, 1983b; Cross et al, 1983; Iversen et al, 1983), although increased levels of dopamine were observed in certain samples of temporal cortex by Iversen et al (1983). Furthermore, the Swedish group have also reported reduced levels of HVA in the CSF of SDAT subjects, whereas most other groups have observed normal levels (chapter 4, section I). One possible explanation could be that the Swedish group did not perform detailed neuropathology on many of their patients. This raises two doubts (Davies, 1983); first, do the so-called SDAT patients actually have the disease? and second, do the patients have signs of Parkinsonian type changes in the substantia nigra? Since Parkinson's disease involves a loss of dopaminergic cells (Hornykiewicz, 1973), it would not be surprising to find dopaminergic involvement if Parkinsonian changes were present.

There is a single report that reduced levels of dopamine receptors are found in the caudate nucleus of SDAT subjects (Reisine et al, 1978), although the levels of receptors in the frontal cortex and hippocampus - areas that show numerous pathological changes - were unaltered. It is interesting to note that administration of the dopamine precursor L-Dopa, which has proved so effective in the treatment of the majority of the dopamine-deficient Parkinson's disease patients, appears to have no effect on patients with SDAT (Adolfsson et al, 1982; Ferris et al, 1982) which suggests that the dopamine system may not be significantly involved in SDAT.

TABLE 5  
Involvement of the dopamine system in SDAT

Biochemistry of the dopaminergic system in SDAT <sup>a</sup>

Observation	References	Areas studied
a) DA levels		
reduced	Gottfries, 1980	caudate nucleus
	Gottfries et al, 1983	caudate nucleus
increased	<sup>b</sup> Iversen et al, 1983	temporal cortex
normal	Adolfsson et al, 1979	frontal cortex, hippocampus, caudate nucleus, putamen
	Yates et al, 1979	caudate nucleus, substantia nigra
	Gottfries et al, 1983	hypothalamus
	<sup>c</sup> Iversen et al, 1983	temporal cortex
	Yates et al, 1983b	caudate nucleus, hypothalamus
b) HVA		
reduced	Gottfries, 1969b	caudate nucleus, putamen
	Adolfsson et al, 1979	caudate nucleus, putamen
	Gottfries, 1980	caudate nucleus
	Gottfries et al, 1983	caudate nucleus
increased	Gottfries et al, 1983	cingulate cortex
normal	Cross et al, 1983	frontal, temporal & occipital cortex, hippocampus
	Gottfries et al, 1983	hippocampus, hypothalamus
	Yates et al, 1983b	caudate nucleus
c) DA receptors		
reduced	Reisine et al, 1978	caudate nucleus
normal	Reisine et al, 1978	frontal cortex, hippocampus, putamen

<sup>a</sup> See table 24 for dopaminergic-related parameters in the cerebrospinal fluid

<sup>b, c</sup> Brodmann areas 38 and 21 respectively



b) Neurons containing neurotransmitter-associated amino acids and neuropeptides

i)  $\gamma$ -Aminobutyric acid-containing neurons

$\gamma$ -Aminobutyric acid (GABA) is an amino acid neurotransmitter which is ubiquitous within the CNS and is largely inhibitory to neurons. In the cortex it appears to be associated with intrinsic neurons (Emson and Lindvall, 1979) and may be the transmitter at as many as a third of all synapses in the mammalian brain (Iversen, 1982).

Investigation of the GABA system in postmortem brain is made difficult by the sensitivity of the marker enzyme glutamic acid decarboxylase (GAD) to agonal status (Bowen et al, 1976; McGeer and McGeer, 1976; Perry et al, 1977a, 1982a; Spokes, 1979). Furthermore, the levels of GABA itself have been reported to be unstable and to increase in postmortem brain (see Tarbit et al, 1980).

Despite these drawbacks the GABA system has been studied in SDAT by several groups (table 6) and although reduced levels of GAD have been reported in SDAT (Bowen et al, 1976; Perry et al, 1977a), Perry et al (1977a) reported that in addition to SDAT, reductions in the levels of GAD also occurred in various other mental disorders. In addition, the reductions of GAD observed in SDAT did not correlate with severity of the neuropathological or clinical abnormalities (Perry et al, 1978b, 1981a). In contrast, other groups have reported that, on the whole, levels of GAD appear to be unaltered in SDAT both in postmortem tissue (Davies and Maloney, 1976; Davies, 1979) and biopsy tissue (Spillane et al, 1977).

Measurements of GABA itself indicate that in SDAT levels are normal both in the hippocampus (Tarbit et al, 1980) and in a large number of other cortical and sub-cortical regions of the brain (Rossor et al, 1982b), although significant reductions were described in the temporal cortex (Rossor et al, 1982). However, using a larger series of patients, Rossor et al (1984) observed that reduced levels of GABA occurred not only in the temporal cortex but were also found in the amygdala, hippocampus and frontal cortex of younger but not older SDAT cases. However, using biopsy temporal cortex taken from younger SDAT cases, Smith et al (1983) found no difference between GABA release in SDAT tissue compared to normals during either resting or potassium-stimulated conditions. Furthermore, it has recently been reported that the levels of GABA do not, on the whole, correlate with the neuropathological severity of the disease as judged by the number of senile plaques present (Mountjoy et

TABLE 6

Involvement of neurons associated with amino acid neurotransmitters in SDAT

I) Biochemistry of the GABAergic system in SDAT<sup>a</sup>

Observation	References	Areas studied
a) GAD		
reduced	Bowen et al, 1976 Perry et al, 1977a	prefrontal cortex frontal, temporal, occipital & parietal cortex, hippocampus
normal	Davies, 1979 Davies & Maloney 1976 Spillane et al, 1977 Davies, 1979	hippocampus frontal, occipital & parietal cortex, hippocampus "neocortex" frontal, cingulate & parietal cortex, hippocampus
b) GABA levels		
reduced	Rossor et al, 1982b <sup>b</sup> Rossor et al, 1984	temporal cortex frontal & temporal cortex, hippocampus, amygdala
normal	Tarbit et al, 1980 Rossor et al, 1982b	hippocampus frontal, occipital & parietal cortex, amygdala caudate nucleus, hypothalamus
c) GABA release normal	<sup>*</sup> Smith et al, 1983	temporal cortex
d) GABA receptors		
reduced	Reisine et al, 1978	frontal cortex, caudate nucleus
normal	Reisine et al, 1978 Bowen et al, 1983 Cross et al, 1984	hippocampus, putamen temporal cortex occipital cortex

II) Aspartic acid and glutamic acid containing neurons in SDAT<sup>a</sup>

Observation	References
Normal hippocampal concentrations of both aspartic and glutamic acids	Tarbit et al, 1980
Normal release of both glutamic and aspartic acids in biopsy cortex	<sup>*</sup> Smith et al, 1983

<sup>a</sup> See table 24 for amino-acid related parameters in the cerebrospinal fluid

<sup>b</sup> Younger (<80) cases only

<sup>\*</sup> Biopsy samples



al, 1984).

GABA receptors have been reported to be reduced in the caudate nucleus and frontal cortex of SDAT brains (Reisine et al, 1978) although normal levels have been reported in both temporal (Bowen et al, 1983) and occipital (Cross et al, 1984) cortex. Interestingly, a report of low levels of GABA in the CSF of three presenile Alzheimer-type dementers (Enna et al, 1977) has recently been confirmed using a larger series of patients (Zimmer et al, 1984).

In summary, data concerning the GABA system in SDAT is conflicting and the reductions observed do not appear to be specific to SDAT. In addition, the sensitivity of the GABA system to non-specific variables makes the interpretation of data difficult.

#### ii) Glutamic and aspartic acids

In addition to GABA, glutamic acid and aspartic acid are also amino acid neurotransmitters within the CNS. These systems have been little studied in SDAT (table 6) due to the lack of specific marker enzymes and difficulty in distinguishing the metabolic and transmitter pools of amino acids. Nevertheless, postmortem measurements of glutamic and aspartic acids in the hippocampus demonstrated no difference in levels between normal and SDAT cases (Tarbit et al, 1980). Furthermore, using biopsy temporal cortex, Smith et al (1983) have shown that the release of glutamic and aspartic acids was not significantly different between SDAT and control cases under both resting and potassium-stimulated conditions.

In addition levels of both glutamic and aspartic acid are unaltered in the CSF of SDAT patients compared to the normal (Bowen, 1983).

#### iii) Neuropeptides

Over thirty different peptides have now been described in the CNS and a variety of these have been studied in SDAT (table 7). To date the only consistent finding is of reduced levels of somatostatin-like immunoreactivity (SLI). However, whereas Rossor et al (1980a) only found reductions in temporal cortex, Davies and colleagues (Davies et al, 1980; Davies and Terry, 1981) observed widespread reductions throughout the cortex. This discrepancy could be due (Davies, 1983) to the more severe cases used by Davies' group particularly since Perry et al (1981a) only observed significant reductions of SLI in the temporal cortex of more severely affected cases.

Vasopressin has been suggested to play a role in memory function

TABLE 7

Neuropeptide levels in SDAT<sup>a</sup>

Observation	References	Areas
<u>1) Somatostatin</u>		
reduced	Davies et al, 1980 Rossor et al, 1980a Davies & Terry, 1981	frontal, temporal & parietal cortex, hippocampus temporal cortex frontal, temporal, cingulate, occipital & parietal cortex, hippocampus
	<sup>b</sup> Perry et al, 1981a Wood et al, 1983	temporal cortex frontal cortex
	Ferrier et al, 1983	frontal, temporal & parietal cortex
	Rossor et al, 1984	frontal & temporal cortex
increased	Ferrier et al, 1983	substantia innominata
normal	Rossor et al, 1980a Ferrier et al, 1983	frontal & parietal cortex, hippocampus, amygdala cingulate cortex, hippocampus, amygdala
<u>2) substance P</u>		
reduced	Crystal & Davies, 1982	frontal, temporal, occipital & parietal cortex
increased	Ferrier et al, 1983	putamen
normal	Perry et al, 1981a Ferrier et al, 1983	hippocampus frontal, temporal, cingulate & parietal cortex
	Yates et al, 1983a	hippocampus, amygdala, substantia innominata frontal, temporal, cingulate & parietal cortex amygdala, caudate nucleus, hypothalamus
<u>3) cholecystokinin</u>		
reduced	<sup>b</sup> Perry et al, 1981a,d	entorhinal cortex
normal	Rossor et al, 1982b	frontal, temporal & parietal cortex, hippocampus caudate nucleus
	Ferrier et al, 1983	frontal, temporal, cingulate & parietal cortex hippocampus, amygdala, substantia innominata
<u>4) vasoactive intestinal polypeptide</u>		
normal	Rossor et al, 1980b	frontal, temporal, occipital & parietal cortex hippocampus, caudate nucleus
	Perry et al, 1981a,d Ferrier et al, 1983	entorhinal cortex frontal, temporal, cingulate & parietal cortex hippocampus, amygdala, substantia innominata
<u>5) neurotensin</u>		
reduced	Ferrier et al, 1983	septum
normal	Biggins et al, 1983 Ferrier et al, 1983	amygdala frontal, temporal, cingulate & parietal cortex hippocampus, amygdala, substantia innominata
<u>6) arginine vasopressin</u>		
reduced	Rossor et al, 1980c	globus pallidus
normal	Rossor et al, 1980c	hypothalamus, locus coeruleus, substantia nigra
<u>7) thyrotropin releasing hormone</u>		
normal	Biggins et al, 1983 Yates et al, 1983a	amygdala cingulate cortex, amygdala, hypothalamus substantia nigra
<u>8) neuropeptide Y</u>		
increased	Allen et al, 1984	substantia innominata
normal	Allen et al, 1984	frontal, temporal, cingulate & parietal cortex hippocampus, amygdala, septum
<u>9) Met-enkephalin</u>		
normal	Rossor et al, 1982a	frontal, temporal & parietal cortex, hippocampus
<u>10) luteinizing-hormone releasing hormone</u>		
normal	Yates et al, 1983a	amygdala, hypothalamus

<sup>a</sup> See table 24 for neuropeptide levels in the cerebrospinal fluid<sup>b</sup> Severe cases only



(DeWeid, 1976; Weingartner et al, 1981) and in this context it is interesting to note that in SDAT (which is characterised by a profound disturbance of memory processes) levels of vasopressin are, on the whole, unaltered in SDAT in the non-cortical areas studied by Rossor et al (1980b). Furthermore, in SDAT there appears to be no major consistent pathological involvement of either cholecystikinin, met-enkephalin, neurotensin, substance P, thyrotropin and luteinizing-hormone releasing hormones or vasoactive intestinal polypeptide (table 7) and although neuropeptide Y has recently been reported to be elevated in the substantia innominata of SDAT subjects (Allen et al, 1984), normal levels were observed in the cortical areas studied. Thus it would appear that there is no gross involvement of neuropeptide-containing neurons in the pathological processes of the disease although, as Davies has pointed out (Davies, 1983), the concentration of the neurotransmitter itself is probably the least stable element of the transmitter system. However studies of the activity of the activity of peptide degrading enzymes also show no profound alterations in peptide metabolism. Hence, although increased activities of a dipeptidase have been reported (Pope et al, 1964, 1965), no consistent alterations in the activities of either angiotensin converting enzyme (Arregui et al, 1982) or various other peptidases (Atack et al, 1983a) have been reported.

Since peptides and the classical neurotransmitters have been shown to co-exist within the same neuron (Hokfelt et al, 1980) it has been suggested that SLI and ACh may be found in the same neuronal structures which degenerate in SDAT leading to a loss of both transmitters (Davies et al, 1980; Davies and Terry, 1981). It is therefore interesting to note that, although AChE and SLI were not observed in the same neurons of the rat striatum (Vincent et al, 1983b), they were observed in the same neurons of cultured embryonic rat cerebrum (Delfs et al, 1984). However, co-existence would seem unlikely for several reasons: First, loss of ChAT can occur without loss of SLI in the earlier cases of SDAT (Perry et al, 1981a); second, whereas the loss of AChE and ChAT is greatest from the upper cortical layers (chapter 2), loss of SLI is greatest from the lower cortical layers (Perry et al, 1983a); third, although a loss of both ChAT and SLI has been reported in the cortex, in the nucleus of Meynert loss of ChAT activity is accompanied by an increase rather than a decrease in SLI (Ferrier et al, 1983) and fourth, surgical lesions of the basal forebrain region of the rat analogous to the primate nucleus of Meynert reduced levels of ChAT in the cortex but



not SLI and also kainic acid lesions of the hippocampus reduced levels of SLI but not ChAT (McKinney et al, 1982a). These observations therefore suggest that ACh and SLI do not co-exist in either the cortex, hippocampus or nucleus of Meynert.

In summary, it would appear that in SDAT there is no major pathological involvement of the peptides studied to date.

### 8. Experimental pharmacology of SDAT

The results discussed in the previous section suggest that, to date the major biochemical abnormality observed in SDAT is a central cholinergic deficit. Since a loss of memory is a major clinical feature of SDAT, the following discussion will describe a) the role of the cholinergic system in memory and b) the pharmacological treatment of SDAT patients with drugs designed to enhance memory, and in particular those directed against the central cholinergic system.

#### a) The cholinergic system in memory processes

The central cholinergic system has been implicated in a wide range of behaviours (see Karczmar, 1976; Butcher and Woolf, 1982) amongst which a central role for the cholinergic system in memory and learning has been demonstrated in numerous animal experiments (for example Deutsch, 1971, 1983; reviewed by Overstreet, 1984). Evidence linking the cholinergic system and memory in man is more difficult to obtain than in other species because of the obvious experimental limitations. However, the use of pharmacological agents directed against the cholinergic system have been shown to alter human memory (for reviews see Davis and Yamamura, 1978; Bartus et al, 1982; Brinkman and Gershon, 1983; Greenwald and Davis, 1983). Thus, administration of the cholinergic antagonist scopolamine impairs learning and produces a disruption in memory performance similar to that seen in SDAT (Drachman and Leavitt, 1974; Drachman, 1977). These scopolamine-induced memory deficits are partially, and reliably reversed by the anticholinesterase physostigmine, but not the CNS stimulant amphetamine, indicating that the effects of scopolamine are due to a specific effect on the cholinergic system rather than a non-specific effect on arousal (Drachman, 1977). In addition administration of physostigmine alone has been shown to facilitate memory performance in both normal young and elderly subjects (Davis et al, 1978,



1979) and pathologically, physostigmine has also been shown to improve memory in amnesia caused by herpes simplex encephalitis (Peters and Levin, 1977). Further evidence of cholinergic involvement in human memory has come from studies using arecoline, a central muscarinic agonist which has been shown to enhance memory when administered to young subjects (Sitaram et al, 1978).

It therefore appears that not only is the cholinergic system involved in memory processes but also pharmacological impairment of the cholinergic system gives a deficit similar to that seen in senile dementia (Drachman and Leavitt, 1974; Drachman, 1977). These observations in conjunction with the reduction in activity of the central cholinergic system in SDAT have lead to the cholinergic hypothesis of the memory disorder associated with SDAT (Smith and Swash, 1978). Consequently, many recent clinical trials have been conducted in order to try and enhance central cholinergic activity, and therefore improve the memory performance, of patients suffering from SDAT and the various approaches used are discussed in the following section.

#### b) Pharmacological treatment of the memory deficit in SDAT

Three approaches have been adopted in clinical trials attempting to enhance central cholinergic activity and therefore memory function in SDAT (for reviews see Bartus et al, 1982; Greenwald and Davis, 1983). First, there are clinical trials that attempt to stimulate the synthesis and release of ACh by providing abundant amounts of the precursor substances choline or lecithin, which is a natural source of choline (and are therefore analogous to the administration of L-Dopa in Parkinson's disease). Secondly, there are those that attempt to enhance cholinergic activity by prolonging the effects of ACh within the synapse by inhibiting the hydrolytic activity of acetylcholinesterase and thirdly, treatments have been carried out in which drugs that act directly at the postsynaptic receptor site as cholinergic agonists have been administered. Of the three, the precursor loading strategy has proved most popular, with doses of choline salts or lecithin being administered by injection or in the diet, yet little or no consistent improvement in SDAT patients has been observed in numerous studies (see Bartus et al, 1982; Corkin et al, 1982; Greenwald and Davis, 1983). However the fundamental requirement upon which the precursor loading therapy is based, namely the ability of peripherally administered precursors to stimulate central acetylcholine synthesis and release remains

controversial (see Bartus et al, 1982; Flentge and van den Berg, 1982). Furthermore, since choline is a weak muscarinic agonist with a potency of 10% relative to ACh (Krnjevic et al, 1982), any effects of increased brain choline levels may be due to effects at the receptor rather than via alterations in ACh synthesis.

In contrast to the precursor loading strategy, clinical trials in which the activity of the central cholinergic system is enhanced by the use of either anticholinesterases or cholinergic agonists appears to be more successful (reviewed by Brinkman and Gershon, 1983). Thus, the use of the anticholinesterase physostigmine has been reported to enhance memory performance in SDAT (Davis et al, 1979, 1983; Christie, 1982; Davis and Mohs, 1982), whilst the anticholinesterase tetrahydro-aminoacridine (THA), which has a longer half-life than physostigmine, was observed to cause a general global improvement in SDAT patients (Summers et al, 1981). The use of arecoline, which acts as a muscarinic agonist rather than an anticholinesterase, has also been reported to enhance memory in SDAT patients, although the improvement was not dramatic (Christie, 1982).

The simultaneous use of both anticholinesterases and lecithin has been reported to improve memory performance in SDAT (Peters and Levin, 1979; Kaye et al, 1982; Thal and Fuld, 1983). Similarly, lecithin applied in conjunction with piracetam - which increases the efficiency of metabolic processes of hypoxic neurons and may therefore act as a "metabolic enhancer"; see Bartus et al, 1982 - also appears to improve the memory performance of SDAT subjects (Ferris et al, 1982).

Although relatively few studies have been conducted using non-cholinergic agents to enhance memory processes, trials based upon the effects of vasopressin on memory (DeWeid et al, 1976; Weingartner et al, 1981) have been carried out. However, vasopressin appears to have little, if any, effect on the limited number of SDAT cases studied so far (Chase et al, 1982; Tinklenberg et al, 1982) which is perhaps not suprising considering the apparent lack of involvement of this peptide in SDAT (section 7.b.iii).

Although the initial clinical trials based on the cholinergic hypothesis have proved disappointing, they in no way invalidate the cholinergic hypothesis of the memory disorder in SDAT. These trials do, however, illustrate the methodological shortcomings of a precursor loading strategy whose in vivo stimulatory effects on ACh synthesis remain to be unequivocally proven, and the use of cholinergic agents, for



example arecoline and physostigmine, which have only relatively short durations of action (Greenwald and Davis, 1983). Consequently, the use of cholinomimetics that can chronically elevate central cholinergic function, and the combination of different treatments should be well worth pursuing (Davis et al, 1982b).

## PART II

ASPECTS OF THE CHOLINERGIC SYSTEM, PARTICULARLY CHOLINESTERASES

The preceding part of the introduction described various aspects of senile dementia of the Alzheimer-type (SDAT) and showed that, to date, a central cholinergic deficit is the major consistent biochemical feature of the disease. Part II of the introduction describes various features of cholinergic neurotransmission, in both the peripheral and central nervous systems, concentrating in particular on characteristics of acetylcholinesterase and butyrylcholinesterase. Both of these enzymes assume particular importance in the experimental treatment of SDAT since they are the enzymes affected by the pharmacological administration of anticholinesterases.



### 1. Biochemistry of the cholinergic synapse

In the present section, various biochemical features of the cholinergic synapse will be briefly described. More detailed discussions of certain aspects of the biochemistry of cholinergic synapses can be found elsewhere (Silver, 1974; Browning, 1976; Rossier, 1977).

The synthesis of acetylcholine occurs in the presynaptic nerve terminal (figure 2) and the substrates for the reaction are acetyl-CoA, derived from glucose metabolism and choline, the latter of which may be derived from the blood in the form of free choline or phospholipid-bound choline or alternatively may be derived from ACh that has been hydrolysed in the synapse and taken up into the presynaptic nerve terminal by the high-affinity choline uptake system (see below). The synthetic reaction is thought to occur in the cytosol and involves the transfer of the acetyl-CoA acetyl group to the choline hydroxyl group and is catalysed by choline acetyltransferase (ChAT; E.C. 2.3.1.6).

The newly synthesised ACh is then thought to be stored in vesicles which upon the arrival of a presynaptic action potential fuse with the presynaptic membrane and discharge their contents into the synapse. The released ACh diffuses across the synapse and interacts with structures on the postsynaptic membrane called receptors and this interaction leads to membrane conductance changes that result in the postsynaptic membrane becoming either hyper- or hypo-polarised.

Different cholinergic synapses can be distinguished on the basis of their interactions with various pharmacological agents and a major division of receptor types is based upon the different sensitivities to muscarine and nicotine. Thus receptors that interact with nicotine are called nicotinic and those interacting with muscarine are called muscarinic (Dale, 1914). Functionally, nicotinic receptors give rise to fast postsynaptic potentials which reach a peak within a few msec and are complete within 100msec. Muscarinic receptors, however, give rise to slow postsynaptic potentials which take 150 to 500 msec to reach a peak and decay even more slowly (Barrett and Magleby, 1976; Brown, 1983). Nicotinic and muscarinic receptors also differ in that they are selectively blocked by curare and atropine respectively. More recently, three different types of muscarinic receptors have been identified on the basis of their affinities (super-high, high and low affinity) towards the cholinergic agonist pirenzepine (Hammer et al, 1980; Caulfield and Straughan, 1983). In the peripheral nervous system, nicotinic receptors

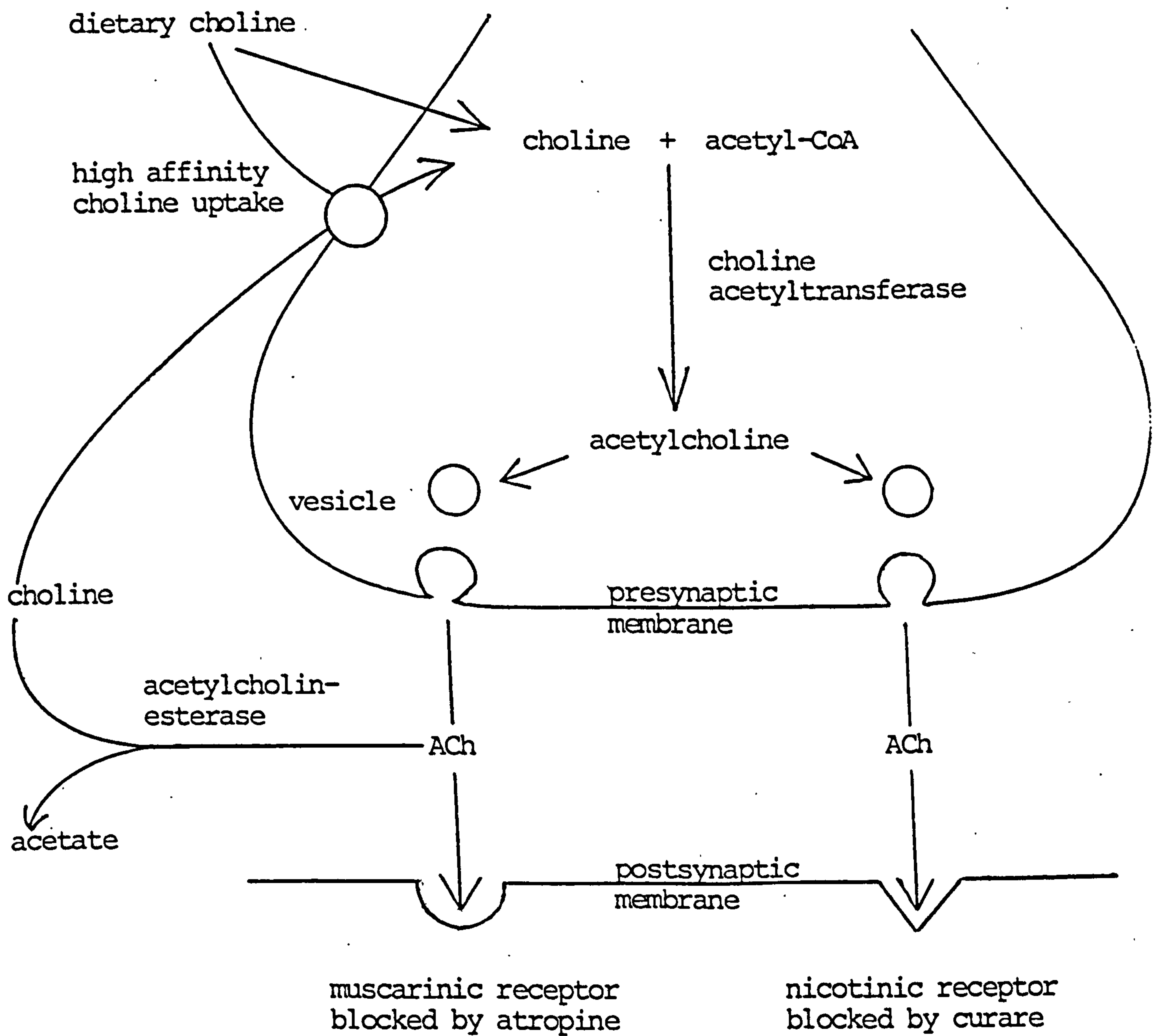


Figure 2. Diagrammatic representation of the basic biochemical features of the cholinergic synapse



occur at the cholinergic synapses between the motor neuron and skeletal muscle and at the synapse between the autonomic preganglionic nerve terminal and the postganglionic cell body, whilst muscarinic receptors are mainly found at the synapse between the postganglionic parasympathetic nerve terminal and the effector organ. In the central nervous system the majority of cholinergic synapses demonstrate predominantly muscarinic characteristics (Krnjevic, 1974; Karczmar, 1976).

The action of ACh within the synapse is terminated by the action of acetylcholinesterase which hydrolyses ACh to acetate and choline. The action of acetylcholinesterase is particularly susceptible to inhibition to organophosphates such as physostigmine. Choline released by the hydrolysis of ACh may be taken back up into the presynaptic nerve terminal by the high-affinity choline uptake system (Kuhar et al, 1973), which is susceptible to inhibition by hemicholinium.

## 2. Anatomy of cholinergic neurons in the peripheral nervous system

The peripheral nervous system consists of two major divisions based upon the direction of nervous conduction. Thus the afferent or sensory nerves carry information about the internal and external environment of the body to the central nervous system (CNS), whilst the efferent nerves carry information from the CNS to the effector organs (figure 3).

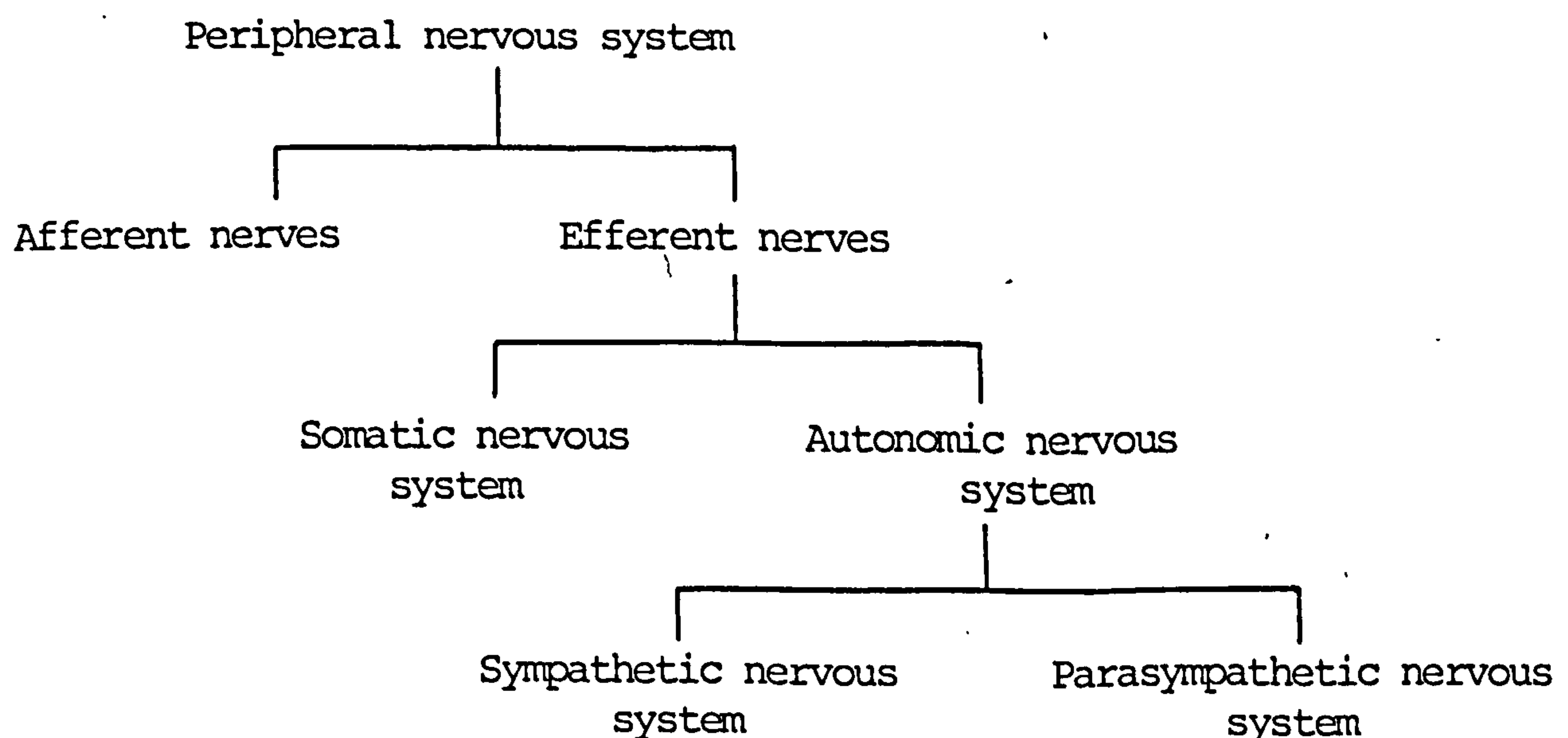


Figure 3. Diagram of the various divisions of the peripheral nervous system

The efferent nerves can be further divided into somatic or autonomic classes. The somatic nerves supply the striated muscle and are responsible for voluntary movements. The autonomic nerves supply the smooth (plain) muscle of organs such as the heart, gut and blood vessels as well as innervating secreting glands. In addition to the functional differences between the somatic and autonomic nervous systems, there is also a major structural distinction. Thus, the projection from the CNS to the effector organ is made by one neuron in the somatic nervous system and two neurons in the autonomic nervous system. Hence, in the autonomic nervous system, one cell body lies within the CNS and its axon (the preganglionic fibre) projects to a ganglion where it synapses with the cell body of a second neuron that in turn sends its axon to the effector organ. The autonomic system itself can also be divided anatomically on the basis of the origin of the preganglionic nerve cell within the CNS and the site of the ganglion. Thus, the parasympathetic division of the autonomic nervous system originates in the brain stem (cranial nerves III, VII, IX and X) and lower (sacral) spinal cord and has long preganglionic fibres which synapse at ganglia near the effector organ whilst the sympathetic division originates in the intermediate regions of the spinal cord (thoracic and lumbar areas) and has short preganglionic fibres which synapse within the sympathetic trunk of ganglia that lie adjacent to the spinal cord.

ACh has been demonstrated to be a neurotransmitter at the somatic nervous system neuromuscular junction and at both the sympathetic and parasympathetic ganglia (figure 4; see also Kuhar, 1976). The

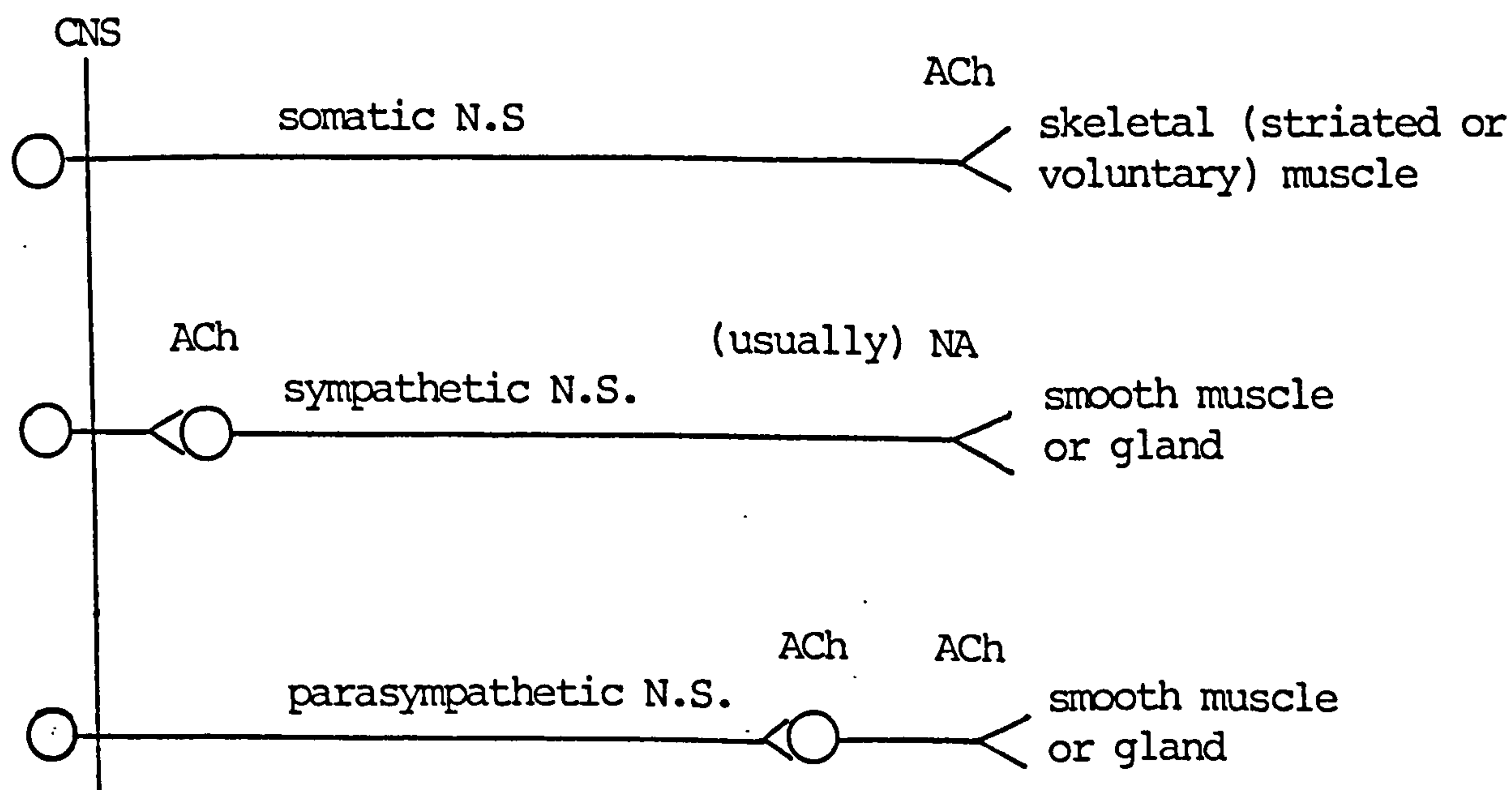


Figure 4. Sites of cholinergic neurotransmission in the peripheral nervous system



sympathetic and parasympathetic divisions do, however, differ in that whilst ACh is also the transmitter at the parasympathetic postganglionic fibre synapse with the effector organ, the sympathetic postganglionic transmitter is usually noradrenaline (except, for example, the sympathetic nerve fibres to sweat glands, which are cholinergic).

### 3. Anatomy of cholinergic neurons within the central nervous system

The following discussion describing the anatomy of the central cholinergic system and is divided into three sub-sections dealing with; a) the methods used for the localization of cholinergic pathways within the central nervous system (CNS), b) the anatomy of central cholinergic pathways projecting to non-neocortical regions and c) the source (primarily the nucleus of Meynert) of the afferent cholinergic input to the neocortex.

#### a) Use of biochemical "markers" to map central cholinergic neurons

Histochemically, the absence of routine, workable methods for the localization of ACh and ChAT (see Butcher and Woolf, 1982), has meant that the localization of cholinergic neurons has, until the recent development of ChAT-specific antisera and its subsequent use in immunohistochemical procedures (reviewed by Wainer et al, 1984), relied upon AChE as a histochemical marker for central cholinergic neurons (for example Lewis and Shute, 1967; Shute and Lewis, 1967). Unfortunately, however, AChE is not necessarily specific for cholinergic neurons (Silver, 1974; Lehmann and Fibiger, 1979) since it is also found in areas with relatively little cholinergic activity - the latter being judged by the content of the synthetic enzyme, choline acetyltransferase - such as the dopaminergic neurons of the substantia nigra (Silver, 1974; Butcher et al, 1975; Lehmann and Fibiger, 1978; Pavlin, 1983), the noradrenergic neurons of the locus coeruleus (Lewis and Schon, 1975; Albanese and Butcher, 1980), the serotonergic raphe nuclei (Butcher and Woolf, 1982) and the cerebellum (Bull et al, 1970; Silver, 1974).

In general, however, with the exception of the regions mentioned above, the regional distribution of AChE and ChAT parallel each other (Kuhar, 1976, Rossier, 1977). Similarly, muscarinic receptor binding and high-affinity choline uptake show corresponding regional variations (Kuhar, 1976). Thus, AChE and ChAT have been found to be present in a



wide range of different mammalian brain structures with, in general, high activities associated with the basal ganglia structures of the caudate nucleus, putamen and globus pallidus and lower activities found in areas of the cortex (Kuhar, 1976; see table 9).

Despite its association with some non-cholinergic neurons, AChE has, nevertheless, proved an invaluable tool in the elucidation of central cholinergic pathways and the presence of AChE along with at least one other cholinergic marker (for example the high-affinity choline uptake system, ChAT, ACh; see Kuhar, 1976; Butcher and Woolf, 1982) is usually sufficient to demonstrate, as far as is possible, the presence of a cholinergic synapse. In order to identify putative central cholinergic pathways, these markers are often measured in a particular target structure after lesioning of a suspected cholinergic tract. Depletions of the cholinergic parameter of interest indicate that the lesion was probably placed in a cholinergic pathway. Using such methods, allied to combined retrograde-tracer and histochemical techniques, data has accumulated to suggest the presence of several central cholinergic pathways which will now be described (for more detailed discussion see Fibiger, 1982).

#### b) Central non-neocortical cholinergic pathways

The best characterized cholinergic tract within the CNS is probably the projection from the septum to the hippocampus. This pathway was initially identified using AChE histochemistry (Lewis and Shute, 1967; Shute and Lewis, 1967) and the hippocampus has subsequently been demonstrated to contain all the features of a cholinergic synapse i.e. ChAT, ACh, high-affinity choline uptake and ACh receptors (chapter 3, section I; reviewed by Fibiger, 1982). A major cholinergic tract originating in the habenula and terminating in the interpenduncular nucleus - which is thought to contain the highest cholinergic activity of any nucleus in the CNS (Fibiger, 1982) - has been suggested on the basis of studies in which lesions were placed in the interpenduncular nucleus (reviewed by Kuhar, 1976). More recently, however, data has accumulated (reviewed by Fibiger, 1982) suggesting that the interpenduncular nucleus does not contain cholinergic cell bodies (see, however, Houser et al, 1983) but is merely a region through which cholinergic fibres pass. Another region containing very high levels of cholinergic markers is the caudate nucleus which is a major component of the striatum. Lesioning studies have shown that interruption of all known major afferent pathways



to the striatum do not reduce levels of AChE, ChAT or ACh itself and, conversely, destruction of the striatum does not appear to alter levels of ChAT in other brain regions (see Kuhar, 1976; Fibiger, 1982). Furthermore, ChAT-staining cell bodies have recently been identified within the caudate nucleus (Eckenstein and Sofroniew, 1983; Houser et al, 1983; Levey et al, 1983) and it therefore appears that the majority of the cholinergic activity of the caudate nucleus is associated with intrinsic neurons which have their cell bodies and terminals within this nucleus (Woolf and Butcher, 1981). Nevertheless, a portion of the cholinergic activity in this region may be associated with the recently identified substantia innominata-caudate nucleus anatomical pathway (Arikuni and Kubota, 1984). Additional central structures receiving cholinergic fibres include the olfactory bulb which appears to receive a cholinergic input from the region of the nucleus of the diagonal band (Mesulam et al, 1983); the amygdala which receives the majority of its afferent input from a rather extensive anatomical region in the basal forebrain (Emson et al, 1979; Nagai et al, 1982; Woolf and Butcher, 1982); the thalamus whose cholinergic innervation originates in the two pontine nuclei, the nucleus cuneiformis and the laterodorsal tegmental nucleus; the lateral geniculate nucleus and the superior colliculus both of which receive a major input from the nucleus cuneiformis; whilst the source of hypothalamic cholinergic activity is unknown (for fuller discussion see Fibiger, 1982).

#### c) Afferent cholinergic pathways to the neocortex

In addition to the above mentioned cholinergic tracts, a major source of cholinergic input to the cortex has recently been characterized, and, since a large proportion of the experimental work presented in this thesis is concerned with the cortex, will now be discussed in more detail than the above mentioned pathways.) The presence of an extrinsic source of cortical cholinergic innervation has been demonstrated using two different approaches. First, chronically isolated cortical slabs, produced by undercutting the cortex, have generally been found to result in large (50-80%) decreases in the levels of activity of cortical AChE and ChAT in cats (Hebb et al, 1963; Green et al, 1970), AChE (but not BChE) in rhesus monkeys (Rosenberg and Echlin, 1965) and AChE in the rat (Ulmar et al, 1975). It should be pointed out, however, that despite observing a highly significant loss of AChE, Ulmar et al (1975) observed a non-significant loss of only 10% of ChAT activity and



also no decrease in cortical ChAT activity was observed by McGeer et al (1977) in undercut rat cortex, although the latter authors considered that their slabs were not as completely isolated as those in the previous reports.

The second approach to determining that the cortex receives a major afferent input has utilised neurotoxic lesions of the cortex. Thus, injections of the excitotoxin kainic acid (which selectively destroys neuronal cell bodies leaving afferent axons and terminals intact; Olney et al, 1974) into the cortex of the rat leads to loss of cortical neuronal cell bodies, but not ChAT (Lehmann and Fibiger, 1979; Lehmann et al, 1980) although similar experiments have been reported to reduce cortical ChAT by approximately 30% (Johnston et al, 1981a) indicating that there may be a small population of intrinsic cortical cholinergic neurons. Despite the conflicting evidence of these two sets of data concerning the presence or absence of an intrinsic cortical cholinergic neuronal population (see also chapter 2, section IV.1.d) both observations are compatible with the suggestion that the major portion of the cortical cholinergic activity is derived from neurons that are extrinsic to (i.e. the cell bodies are found outside) the cortex. In addition, the treatment of fetal rat cortex with methylazoxymethanol leads to a subsequent reduction in the intrinsic cortical neuronal population found in the adult rat, yet under such conditions there is an increase in cortical cholinergic activity (Johnston and Coyle, 1979) again suggesting that the majority of the cholinergic activity within the cortex is derived from subcortical regions.

The localization of the subcortical region that is the source of the cortical cholinergic afferent input has recently been intensively studied, and considerable evidence has now accumulated to suggest that not only does an anatomic pathway exist between the neurons lying beneath the anterior commissure in the basal forebrain but that these neurons are cholinergic and are responsible for the major cholinergic input to the cortex. There is considerable interspecies variation in the organisation of these neurons which are intermingled with the globus pallidus in rodents whilst in primates, including man, this nucleus attains its greatest development both in size and in differentiation from adjacent cell groups (Gorrry, 1963). These neurons were first described by Meynert (1872) and have subsequently been called the nucleus of Meynert. The nomenclature is, however, confusing since these neurons have also been called the nucleus of the ansa lenticularis, the nucleus of the ansa



peduncularis, nucleus basalis, preoptic magnocellular nucleus, substantia innominata, and the nucleus of the septal plane (Mesulam and Mufson, 1984). In the present discussion, this region will be called the nucleus basalis of Meynert (nbM).

The identification of an anatomical connection between these cells and the cortex has been based on three observations (table 8); firstly, injections of horseradish peroxidase, which is transported retrogradely along axons, labels the cell bodies in this region when injected into the cortex of the monkey, rat, opossum, and shrew Divac, 1975; Kievit and Kuypers, 1975; Jones et al, 1976; Lehmann et al, 1980; Pearson et al, 1983a). Secondly, cortical lesions lead to a retrograde degeneration of neurons in the nbM region of the rabbit (Das, 1971) rat (Sofroniew et al, 1983) and humans and monkeys (Gorry et al, 1963; Jones et al, 1976; Pearson et al, 1983b) and thirdly, injections of radiolabelled amino acids into the region of the rat analagous to the primate nbM resulted in the detection of label in the cortex, presumably transported by a basal forebrain-cortex pathway (Fibiger, 1982).

The cholinergic nature of this anatomical pathway has been demonstrated using a number of techniques; first, the cholinergic nature of the nbM neurons has been established in the rat, monkey and human using AChE histochemistry (Shute and Lewis, 1967; Wenk et al, 1980, Candy et al, 1981; Perry et al, 1982b, 1984b; Rossor et al, 1982c; Mesulam et al, 1983; Satoh et al, 1983; Parent et al, 1984; figure 5) and more recently ChAT immunocytochemistry (Armstrong et al, 1983; Eckenstein and Sofroniew, 1983; Hedreen et al, 1983; Houser et al, 1983; Levey et al, 1983; Nagai et al, 1983; Pearson et al, 1983c; Satoh et al, 1983; McGeer et al, 1984). In addition, biochemical microdissection techniques have also confirmed that the neurons in this region are cholinergic as judged by their high ChAT activity (Candy et al, 1981; Davies and Feisullin, 1982; McKinney et al, 1982b; Henke and Lang, 1983; Perry et al, 1984b). Second, injections of tracers into the cortex retrogradely label cells in the nucleus basalis region which are also AChE-rich (Mesulam and van Hoesen, 1976; Lehmann et al, 1980; Bigl et al, 1982; Mesulam et al, 1983) and stain immunohistochemically for ChAT (Woolf et al, 1983; Wahle et al, 1984).

Whilst these techniques clearly demonstrate that neurons of the nbM are cholinergic and that the same cholinergic neurons also project to the cortex, they do not, however, indicate the proportion of the cortical cholinergic activity accounted for by this pathway. In order to

TABLE 8

Evidence for a major afferent cortical cholinergic input from the nucleus of Meynert (nbM)

I) Evidence for an afferent cortical cholinergic input

a) Levels of AChE and ChAT decrease in chronically isolated cortex	Hebb et al, 1963 Green et al, 1970	Rosenberg & Echlin, 1965 Ulmar et al, 1975
b) Injections of kainic acid into the cortex result in little, if any, loss of cortical ChAT	Lehmann & Fibiger, 1979 Johnston et al, 1981a	Lehmann et al, 1980

II) Evidence for an anatomical projection from the nucleus of Meynert to the cortex

a) Injections of horseradish peroxidase into the cortex label cells in the nbM region	Divac, 1975 Kievit & Kuypers, 1976 Mesulam et al, 1983	Jones et al, 1976 Lehmann et al, 1980 Pearson et al, 1983a
b) Cortical lesions lead to retrograde degeneration of neurons in the nbM	Gorry, 1963 Jones et al, 1976 Sofroniew et al, 1983	Das, 1971 Pearson et al, 1983b
c) Radiolabelled amino acids injected into the rat basal forebrain label the cortex	Fibiger, 1982	

III) Evidence for the cholinergic nature of neurons in the nucleus of Meynert

a) The neurons stain histochemically for AChE	Shute & Lewis, 1967 Candy et al, 1981 Rossor et al, 1982c Sato et al, 1983	Wenk et al, 1980 Perry et al, 1982b, 1984b Mesulam et al, 1983 Parent et al, 1984
b) The neurons stain immunohistochemically for ChAT	Armstrong et al, 1983 Eckenstein & Sofroniew, 1983 Hedreen et al, 1983 Levey et al, 1983 Nagai et al, 1983 Sato et al, 1983	Houser et al, 1983 Mesulam et al, 1983 Pearson et al, 1983c McGeer et al, 1984
c) Microdissected tissue from the nbM is rich in cholinergic-related enzyme activities	Candy et al, 1981 McKinney et al, 1982b Henke & Lang, 1983	Davies & Feisullin, 1982 Perry et al, 1984b
d) Retrograde tracers injected into the cortex label cells in the nbM that stain for AChE and ChAT	Mesulam & van Hoesen, 1976 Lehmann et al, 1980 Mesulam et al, 1983 Wahle et al, 1984	Bigl et al, 1982 Woolf et al, 1983

IV) Evidence that the nbM is responsible for a MAJOR afferent cortical cholinergic input

Lesions in the nbM produce large (usually >50%) reductions in cortical AChE and ChAT	Kelly & Moore, 1978 Johnston et al, 1979, 1981a,b Lehmann et al, 1980 Hartgraves et al, 1982	Wenk et al, 1980
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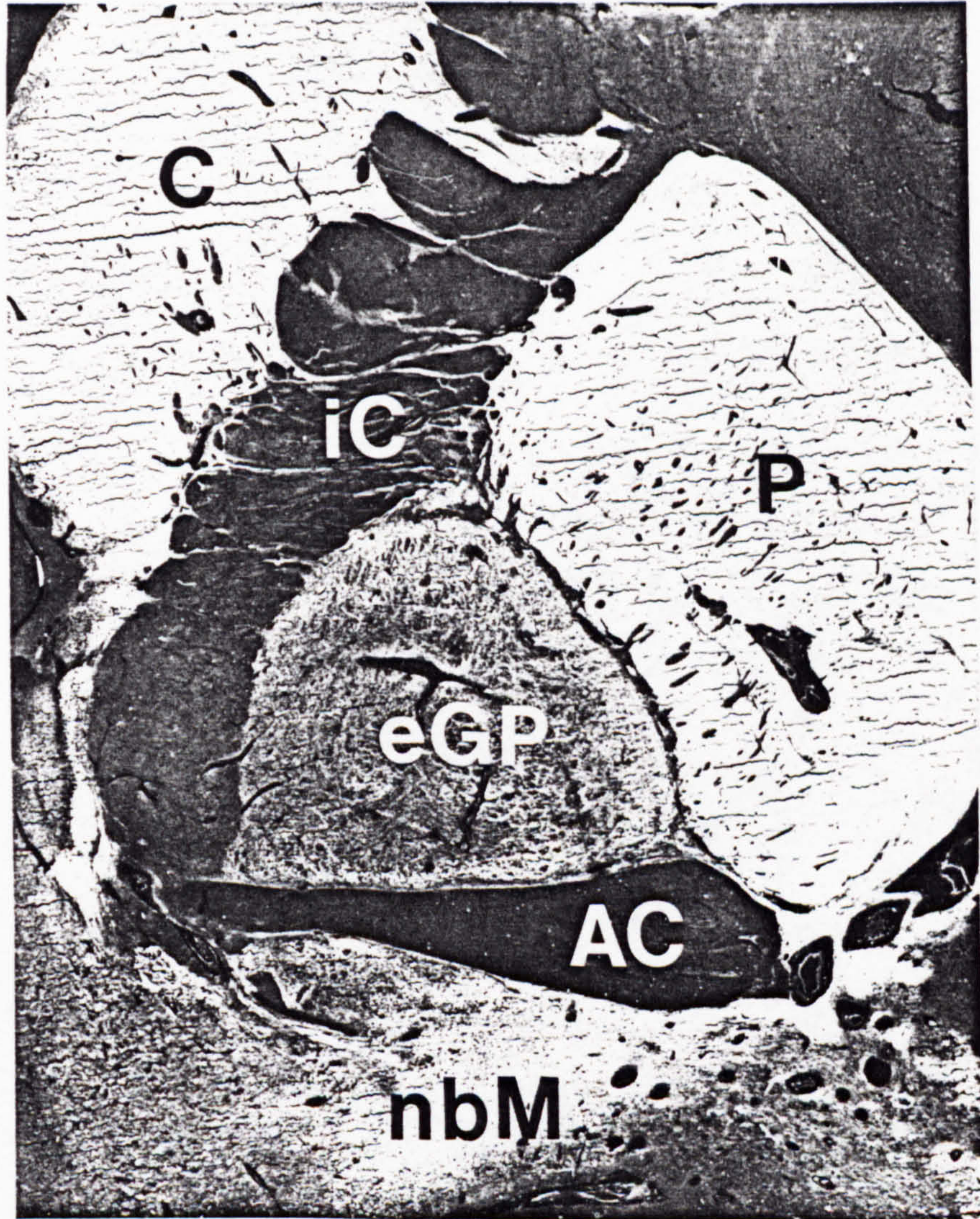


Figure 5. Histochemical demonstration of the AChE distribution (areas containing most activity appear lighter than those areas containing little activity) in the basal forebrain region of the human brain. The nucleus of Meynert (nbM) is clearly visible as an intensely staining region lying beneath the unstained anterior commissure (AC). Other structures include the caudate nucleus (C), external globus pallidus (eGP), internal capsule (iC) and putamen (P).



establish the quantitative contribution of the nbM towards the total cortical cholinergic activity, a third approach has been used. Thus, electrolytic and kainic acid lesions placed in this basal forebrain region have been observed to reduce both cortical AChE, ChAT and high-affinity choline uptake in rats by over 50% in most cases (Kelly and Moore, 1978; Johnston et al, 1979, 1981a,b; Lehmann et al, 1980; Wenk et al, 1980; Hartgraves et al, 1982). The possibility that the nbM may be merely a region through which axons from a more caudal source pass is unlikely since lesions placed slightly caudal to the nucleus basalis produced no alterations in cortical activity (Lehmann et al, 1980).

In addition to the basal forebrain projection, cholinergic pathways to the cortex have recently been described originating in the cuneiformis-pedunculopontine nuclei of the mesencephalic reticular formation in the monkey (Mufson et al, 1982) and in the dorsolateral pontine tegmentum of the rat where substance P is reported to coexist in the cholinergic neurons (Vincent et al, 1983a).

In summary, the presence of a major afferent cholinergic input to the cortex has been demonstrated and it would appear that anatomical evidence exists for a projection from the neurons of the basal forebrain to the cortex. Furthermore, basal forebrain neurons have been demonstrated to possess unequivocal cholinergic activity and lesions of these neurons reduces cortical cholinergic activities by over 50% indicating that the nbM is the major source of cortical cholinergic activity. The experimental observations concerning this cholinergic pathway is supported by the pathological situation that occurs in subjects suffering from SDAT (section I.7.a.i), where a pathological loss or shrinkage of nbM neurons is accompanied by reduced cholinergic activity both within this nucleus and in the cortex.

#### 4. The cholinergic system in the human brain

The study of the central cholinergic system has mainly been carried out using lesioning techniques in the experimental animal. Such studies are, of course, inappropriate for the study of the human central cholinergic system except for the occasional subjects in whom surgical decortication has been performed (Pearson et al, 1983b; see below). To establish whether or not cholinergic pathways exist in man analogous to those seen in other species, it has been necessary to demonstrate that



anatomical and biochemical distributions of cholinergic-associated markers parallel those seen in other species. However, in man the study of these markers is, apart from limited studies carried out on biopsy tissue, restricted to the use of postmortem material which is subject to various non-specific influences not encountered in studies of experimental animals. It is therefore necessary to establish what effect factors such as the time of day of death, agonal status, the delay between death and postmortem (postmortem delay) and tissue storage have upon the parameter under study (reviewed by Perry and Perry, 1983).

a) Effect of non-specific factors on the levels of enzyme activity

Although no data for AChE or BChE exists, the effects of circadian variations on ChAT activity reported by Perry et al (1977c) were not observed by Spokes (1979). The agonal status of the subject appears to have little effect on AChE, ChAT (Bowen et al, 1976; McGeer and McGeer, 1976; Perry et al, 1977a, 1982a; Spokes, 1979) or BChE (Perry et al, 1982a) although ChAT has been reported to be lower after traumatic death (McGeer and McGeer, 1976). In addition, the effect of postmortem delay also appears, on the whole, to have little effect on either AChE or ChAT activities, although ChAT does appear to be less stable than AChE (McGeer and McGeer, 1976; Mackay et al, 1978; Spokes, 1979). Similarly, BChE has also been reported to be stable in human postmortem nervous tissue (Ord and Thompson, 1952). Furthermore, the activity of none of these enzymes appears to be significantly affected by the storage of frozen tissue. Thus, AChE and BChE are stable in human brain tissue stored at  $-20^{\circ}\text{C}$  for at least 6 months (Foldes et al, 1962), whilst ChAT, for which data is lacking in human tissue, was found to be stable for a period of storage of up to 8 months (Spokes, 1979). Also, at temperatures of  $-70^{\circ}\text{C}$  or less, biochemical activities are generally assumed to be stable for several years (see Perry and Perry, 1983).

Another possible influence on enzyme levels is that of drugs. This area has, however, received little attention presumably due to the considerable difficulties in correlating any given parameter with the wide range of drugs and the multiple drug regimes used in treatment of the patient. Spokes (1979) was, however, unable to find any effect of opiate drugs on the levels of ChAT in postmortem brain. It therefore seems that certain components of the cholinergic system are relatively unaffected by various non-specific factors and may therefore be reliably studied in the human postmortem brain.



b) Distribution of cholinergic activities in the human postmortem brain

Table 9 summarizes previous reports on the distribution of the enzymes AChE, BChE and ChAT. The distribution of AChE and ChAT roughly parallels that seen in the rat, monkey and baboon (Kuhar, 1976) with high levels associated with the basal ganglia (caudate nucleus, putamen and globus pallidus) whilst the cerebral cortex appears to have the lowest activities and the limbic structures of the hippocampus and the amygdala have activities approximately twice those of the cerebral cortex. Early studies (Okinaka et al, 1951, 1961; Ord and Thompson, 1952) showed a similar distribution of total cholinesterase (i.e. AChE plus BChE), the majority of which was confirmed as being AChE either biochemically (Ord and Thompson, 1952) or histochemically (Okinaka et al, 1961). The distribution and levels of BChE are discussed separately in section 7.

The use of biopsy material has allowed the demonstration of an active ACh-synthesis in human neocortex (Sims et al, 1980, 1981, 1983a; Bowen et al, 1983) along with a high-affinity choline-uptake system (Sims et al, 1983a). In addition, a metabolically active high-affinity choline uptake system has also been demonstrated in synaptosomes prepared from postmortem brain material, and the relative activity of the hippocampal synaptosomes was twice that of the frontal cortex (Rylett et al, 1983) in agreement with the levels of AChE and ChAT observed in these regions.

There are several lines of evidence indicating that, in the human, there is a major cortical input derived from the nucleus basalis of Meynert (nbM) region of the basal forebrain: First, observations on the AChE histochemistry of human fetal cortex indicates that the cortex receives a cholinergic input from underlying regions (Kostovic, 1979); second, the cholinergic nature of cells in the nbM region have been confirmed (Candy et al, 1981; Perry et al, 1982b, 1984b; Rossor et al, 1982c; see also table 2); third, the anatomical organisation of the nbM region is identical to that seen in the monkey which has been shown to project to the cortex (Mesulam et al, 1983); fourth, in human subjects in which the majority of one hemisphere had been removed, a retrograde cellular degeneration was observed in the nbM (Pearson et al, 1983b) similar to that seen in experimental animals (Sofroniew et al, 1983) and fifth, the pathological changes observed in SDAT (section I.7.a.i) in the cortex and nbM are in agreement with a projection from the nbM to the cortex.



TABLE 9

Distribution of cholinesterases and choline acetyltransferase in the human postmortem brain

	Foldes et al 1962	Domino et al 1973	McGeer & McGeer 1976	Mackay et al 1978	Cote & Kremzner 1983
Average age, years	42	53	42	61	60
Post mortem delay, hours	3-5	<36	7.5	30	9-12
Enzyme studied	AChE    BChE	AChE    BChE	AChE    ChAT	AChE    ChAT	AChE    BChE
BRAIN AREA					
Caudate nucleus (actual activity; see footnote for units)	100	100			
	23	610	100	100	100
			25.7	29	6.7
		10.7		100	100
		9.8		4.2	290
Forebrain: diencephalon					
Thalamus	44	14	12	37	
Hypothalamus	95	16	23	41	
		7.8	6.6	23	
		5.5	7.3	53	
Forebrain: rhinecephalon					
Hippocampus		15	14		
Amygdala		13	25		
Nucleus accumbens			89	38	48
Septal area		31	16	26	110
Fornix		20	13		
			4.6		
Forebrain: basal ganglia					
Putamen			107		18
Globus pallidus	88	94	31		110
			107		
Forebrain: cerebral cortex					
Frontal		5.9	9.8	27	19
Occipital		3.6	5.4	5.4	2.7
Parietal	2.3	3.9	10.7		59
Temporal	3.6	22	9.4	30	
		16	4.4	11	
			5.6	14	
Midbrain and hindbrain					
Pons					
Substantia nigra		10		36	
Cerebellum	15	77	25	43	61
			25	29	11
			6.8		
			3.4		

Actual activity of caudate nucleus homogenates: AChE =  $\mu\text{mol/min/g}$  wet weight tissue; BChE =  $\text{nmol/min/g}$ ; ChAT =  $\text{nmol/min/g}$

### 5. Cholinesterases: nomenclature

The terms "true" and "pseudo" cholinesterase were first used by Mendel and Rudney (1943) to describe the enzymes associated with human erythrocyte membranes and serum respectively. The erythrocyte enzyme hydrolysed ACh in preference to the higher choline esters such as butyrylcholine or propionylcholine and exhibited the phenomenon of inhibition by high substrate concentrations. In contrast, the serum enzyme preferentially hydrolysed the higher choline esters and showed no substrate inhibition phenomenon.

Augustinsson and Nachmansohn (1949) introduced the terms acetylcholinesterase to replace "true" cholinesterase and the term cholinesterase was restricted to the "pseudo" cholinesterase. Subsequently, the Enzyme Commission (1965) recommended the use of acetylcholinesterase and cholinesterase as trivial names with acetylcholine acetylhydrolase (E.C.3.1.1.7) and acylcholine acyl-hydrolase (E.C.3.1.1.8) as respective systematic names. This, however, leaves no collective term to describe an acylhydrolase of unknown substrate specificity and many authors still use cholinesterase as a collective term to describe both acetyl- and pseudo-cholinesterase.

Since the pseudocholinesterase (pseudoChE, also called non-specific cholinesterase) in humans has a greater hydrolytic action towards butyrylcholine than propionylcholine (Myers, 1953) the term butyrylcholinesterase will be used throughout to describe this enzyme (compare other species where the serum enzyme preferentially hydrolyses propionylcholine and is therefore called propionylcholinesterase; Silver, 1974). When the specificity of the enzyme towards the butyryl or propionyl derivative is not apparent or stated, the term pseudoChE will be used. The term acetylcholinesterase is retained to describe the enzyme which preferentially hydrolyses ACh, whilst cholinesterase will be used as a collective term for both types of enzyme. Table 10 illustrates the different properties of these two classes of enzyme (modified from Silver, 1974).

### 6. Hydrolytic mechanisms of cholinesterases

Acyl esterases are enzymes which hydrolyse esters of carboxylic acids and mono- and poly-hydric alcohols. Cholinesterases are



TABLE 10

Properties of the two different classes of choline ester hydrolysing enzymes.<sup>a</sup>

Trivial name	Acetylcholinesterase	Pseudocholinesterase Non-specific cholinesterase
Systematic name	Acetylcholine acetyl- hydrolase; E.C.3.1.1.7	Acylcholine acyl- hydrolase; E.C.3.1.1.8
Reaction catalysed	$\begin{array}{c} \text{acetylcholine} + \text{H}_2\text{O} \\ \downarrow \\ \text{choline} + \text{acetate} \end{array}$	$\begin{array}{c} \text{acylcholine} + \text{H}_2\text{O} \\ \downarrow \\ \text{choline} + \text{corresponding acid} \end{array}$
INHIBITORS		
Physostigmine	inhibited by $10^{-5}$ M	inhibited by $10^{-5}$ M
DFP	inhibited by $10^{-5}$ to $10^{-6}$ M	inhibited by $10^{-7}$ to $10^{-8}$ M
Iso-OMPA	resistant to $10^{-5}$ M	susceptible to $10^{-5}$ M
Ethopropazine	resistant to $10^{-5}$ M	susceptible to $10^{-5}$ M
BW284c51	susceptible to $10^{-5}$ M	resistant to $10^{-5}$ M
EFFECT OF IONS		
Ni + Zn	strong inhibition	weak or no inhibition
Mn + Mg	Mg more effective activator	Mn more effective activator
INHIBITION BY EXCESS SUBSTRATE	yes, concentrations >1-2mM	no

<sup>a</sup> Modified from Silver (1974)

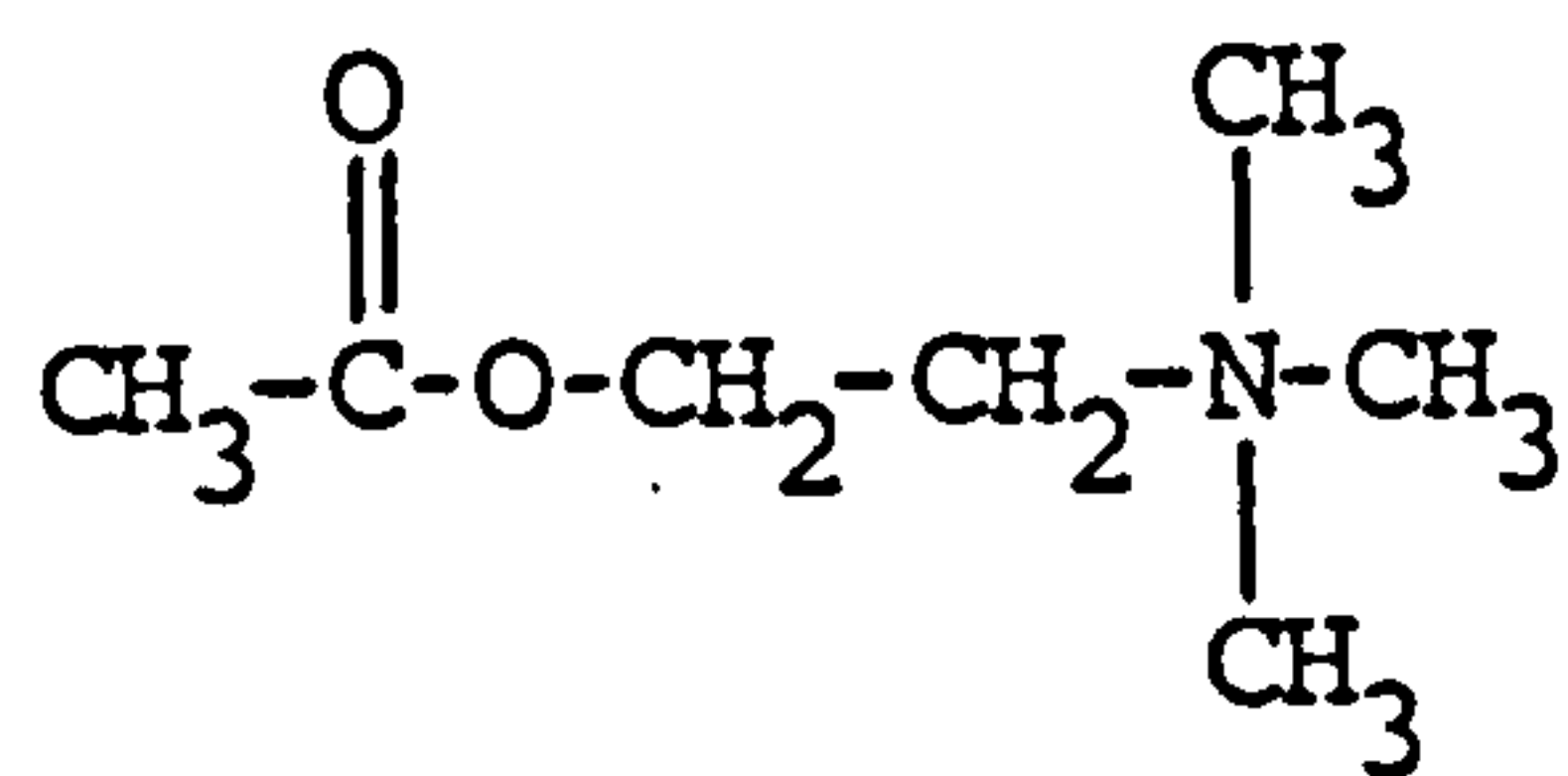
distinguished from other acyl esterases by a marked affinity for esters of the quaternary-ammonium alcohol, choline, and also their complete inhibition by  $10^{-5}$ M physostigmine (eserine). Figure 6 shows the structures of various cholinesterase substrates and physostigmine.

Traditionally attention has been focussed on the mechanism of action of AChE rather than pseudoChE. Thus the following discussion will concentrate on AChE although pseudoChE's are thought to hydrolyse choline esters using a similar mechanism. More detailed discussions are available elsewhere (Silver, 1974; Main, 1976).

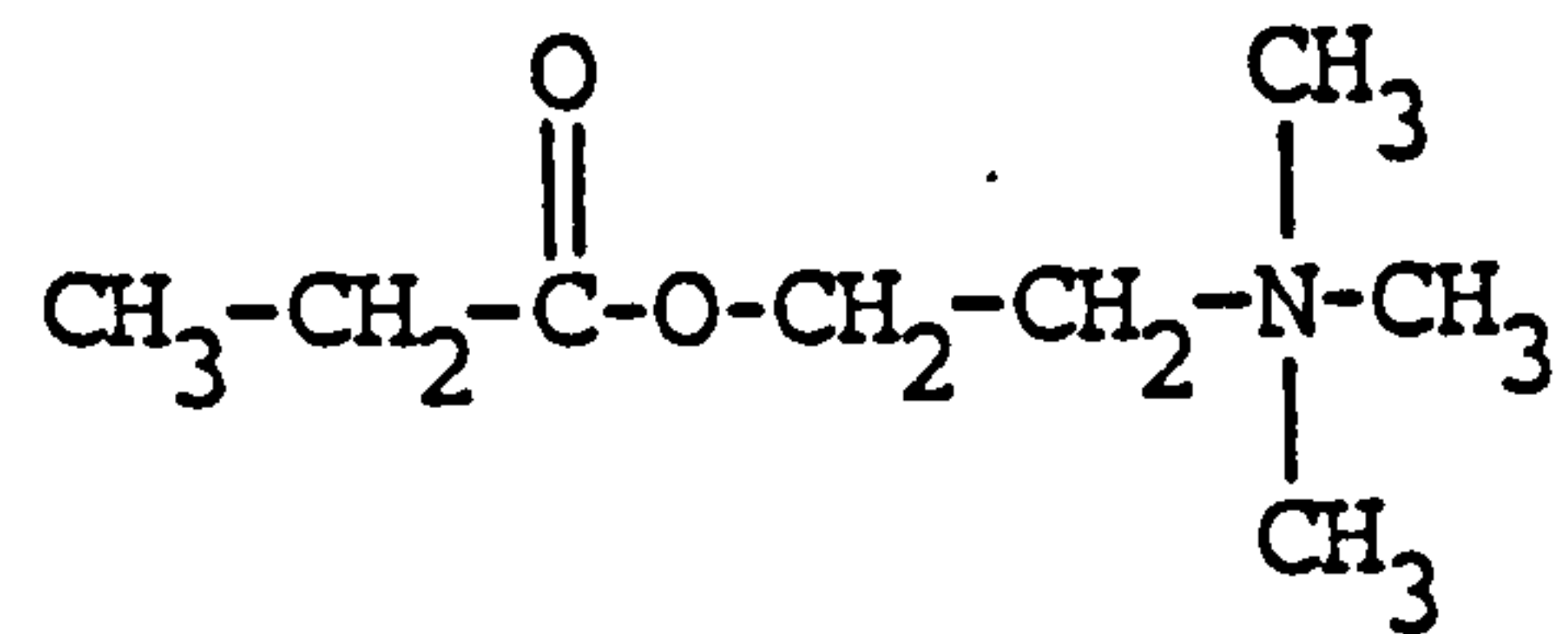
Any theory of the mechanism of action of cholinesterases should not only explain the hydrolytic activity of the enzyme towards choline-esters but should also be able to explain the inhibitory effects of various substances. Since choline esters and certain inhibitors (e.g. dibucaine, edrophonium, BW284c51) contain positively charged quaternary nitrogen atoms, Zeller and Bissegger (1943) postulated the presence of two different sites at the active centre of the AChE enzyme. The first of these is a negatively charged region, the anionic site (which is thought to contain a dissociated glutamic acid residue), that binds to the quaternary nitrogen of either the substrate or inhibitor. The second, esteratic, site is thought to contain serine, histidine and tyrosine residues. Based upon this structure of the active centre, a model of ACh hydrolysis by AChE has been proposed (figure 7) .

In this scheme, the electrostatic bond that forms between the quaternary nitrogen of ACh and the negatively charged glutamic acid residue of the anionic site orientates the molecule so that the carbonyl group of acetylcholine is presented to the esteratic site (figure 7 a). At the esteratic site, the serine hydroxyl oxygen is already activated by hydrogen bonding between the serine hydroxyl hydrogen and the adjacent nitrogen on the imidazole group of the histidine residue. This results in the serine hydroxyl oxygen being more nucleophilic than normal. Although hydrogen bonds form between the carbonyl oxygen of ACh and the imidazole group of histidine, and between the tryosine hydroxyl and the ester linkage of ACh, these are not considered to play a crucial role in the hydrolytic mechanisms (Main, 1976) although they do make the carbonyl carbon of ACh more electrophilic. Under these activated conditions the serine becomes acylated (figure 7 b) with the resultant dissociation of the ester linkage. This acylated enzyme-substrate intermediate is rapidly hydrolysed by water to regenerate the free enzyme and an acetate ion (figure 7 c).

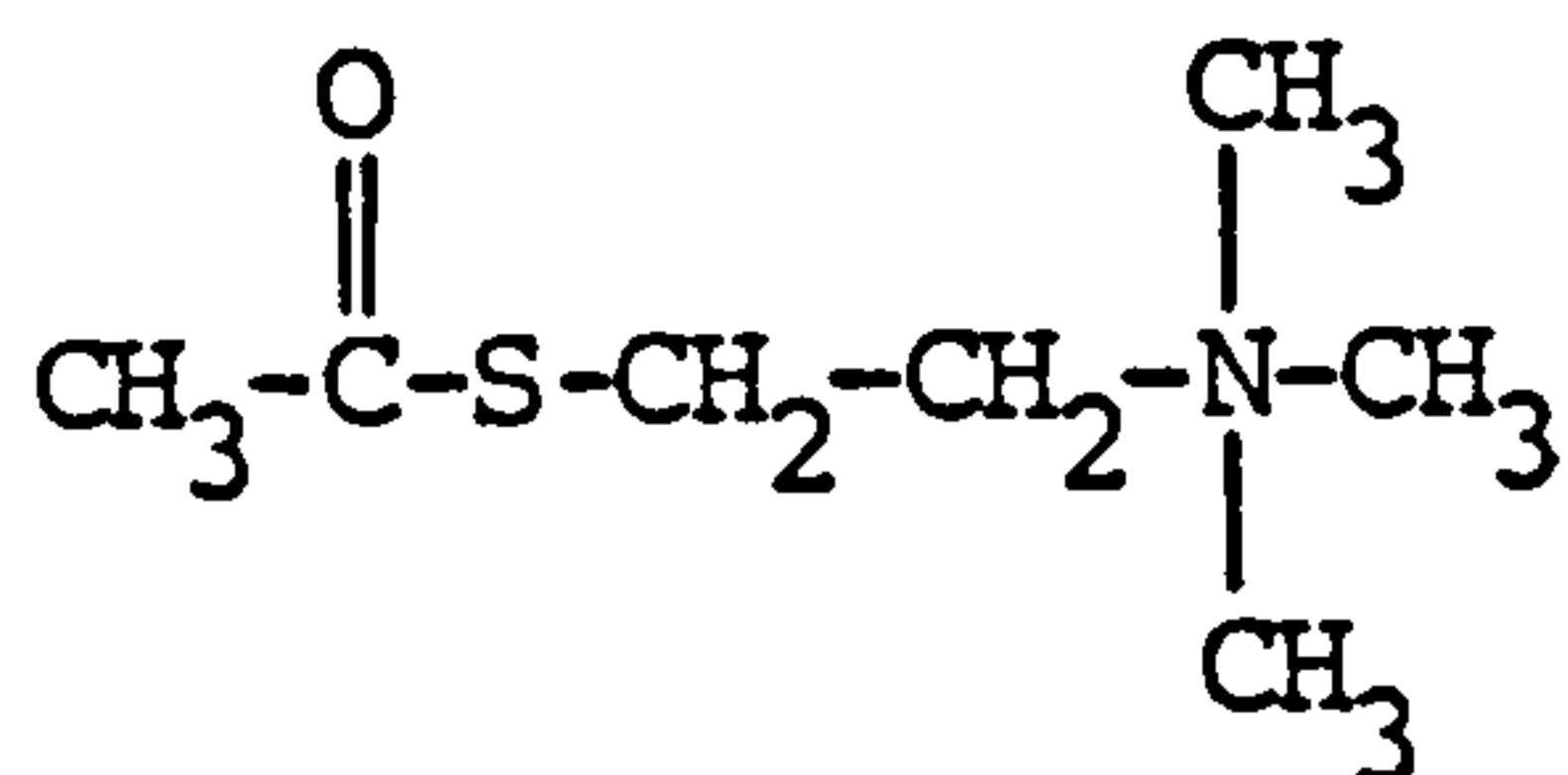




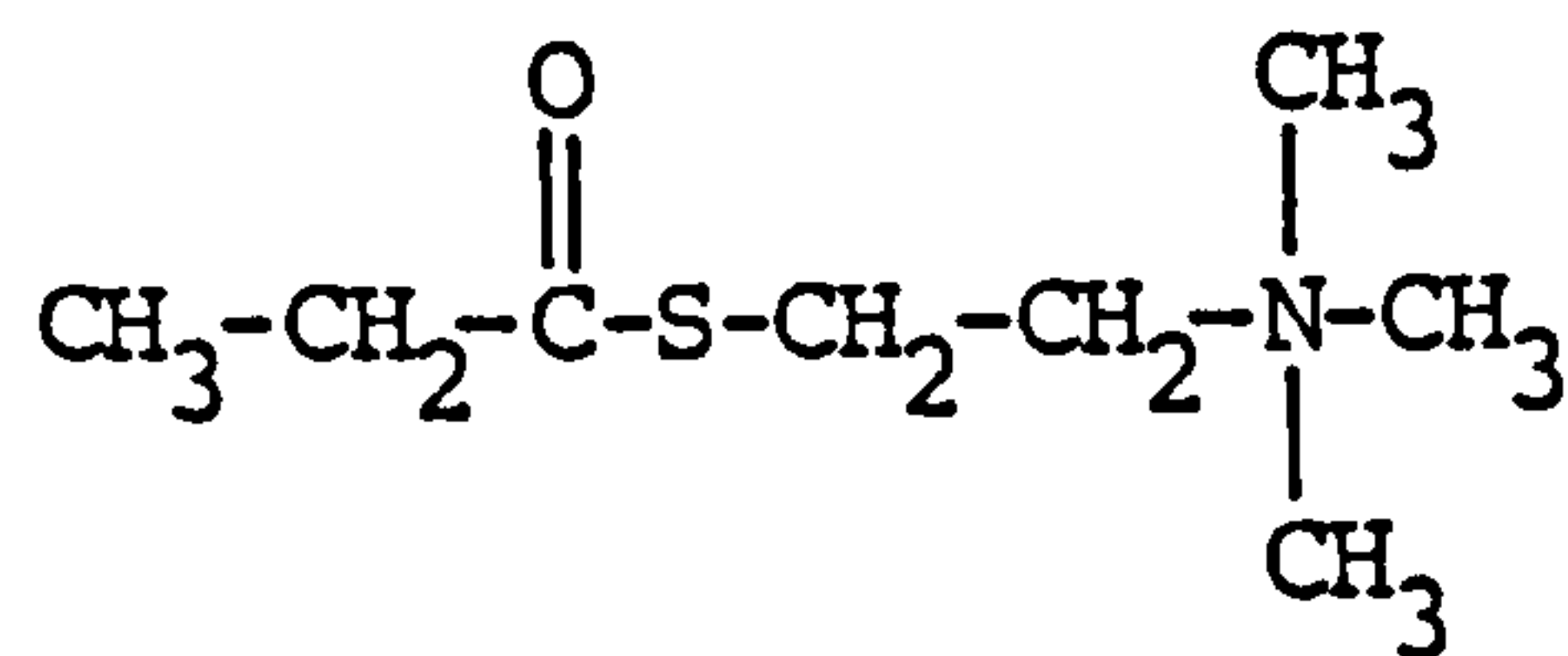
acetylcholine



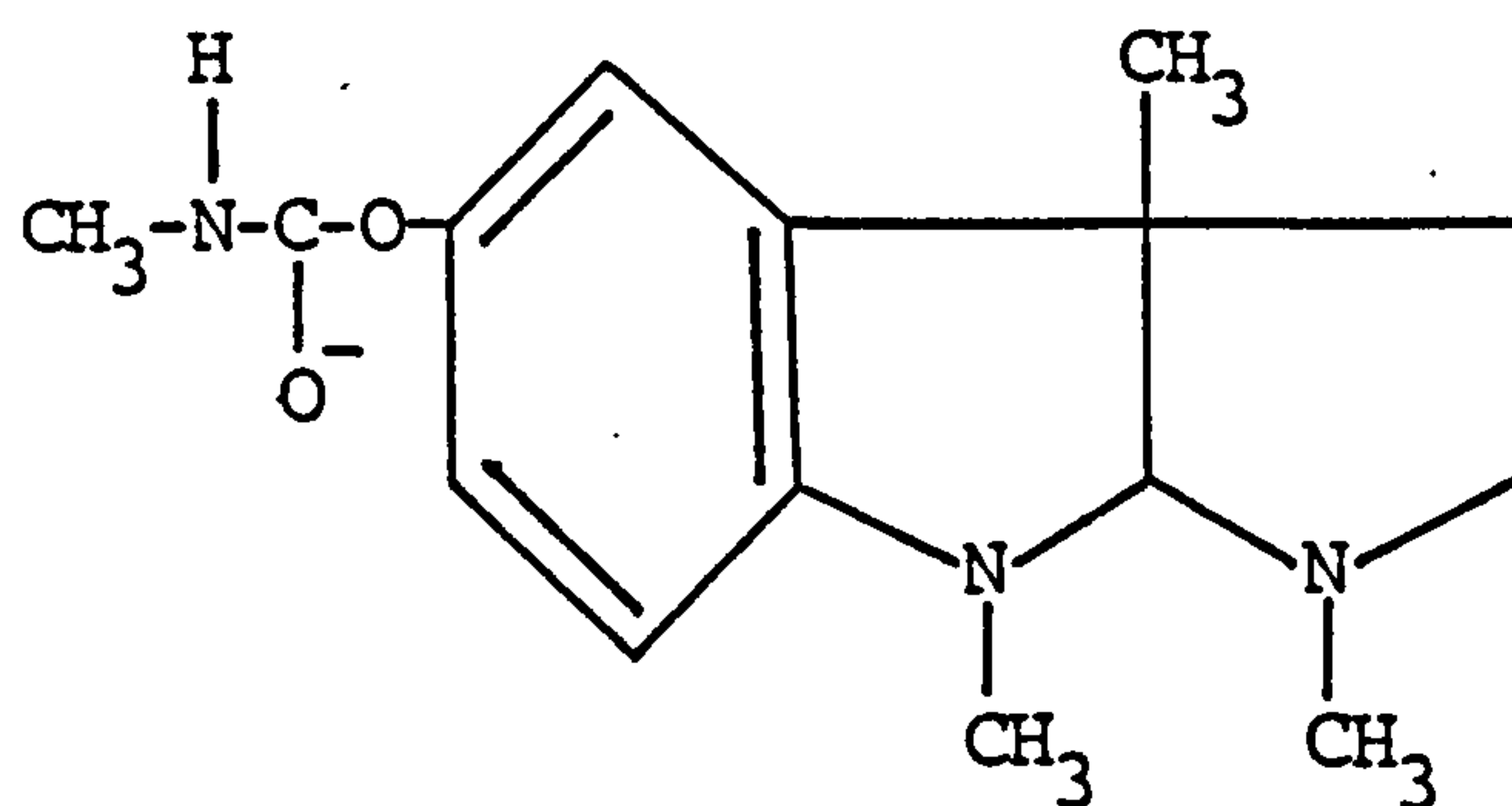
butyrylcholine



acetylthiocholine



butyrylthiocholine



physostigmine

Figure 6. Diagram showing the structures of acetyl-, acetylthio-, butyryl- and butyrylthio-choline plus physostigmine.

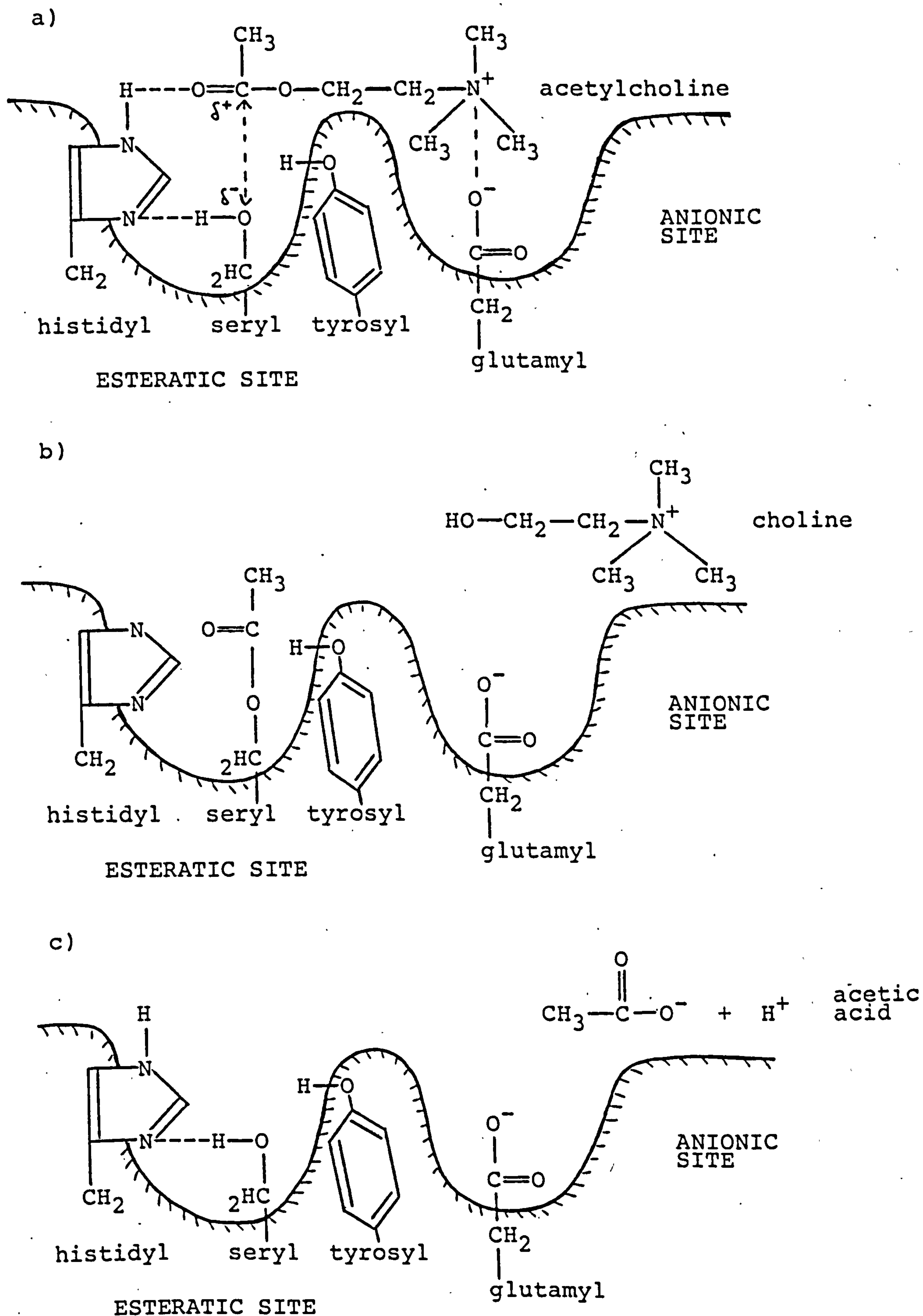


Figure 7. Diagrammatic representation of the hydrolysis of acetylcholine by acetylcholinesterase. a) attraction of acetylcholine to the active centre particularly to the anionic site, b) formation, at the esteratic site, of the unstable acylated enzyme intermediate and release of choline and c) hydrolysis of acylated enzyme yielding acetic acid and unoccupied enzyme. Dashed lines indicate electrostatic attraction.



Almost all reversible inhibitors contain at least one positively charged nitrogen atom (Main, 1976) and it is thought that this group is attracted by, and binds to, the negatively charged anionic site by a combination of electrostatic and Van der Waals forces (Main, 1976). Reversible inhibitors, therefore, act purely via non-covalent forces.

In contrast, irreversible inhibitors act at the esteratic rather than anionic site and form stable, covalently bonded complexes with the enzyme. The majority of the irreversible inhibitors fall into two major classes; the organophosphates and the carbamates. These compounds act by forming stable acyl-enzyme intermediates via their phosphate and carbonyl groups respectively. In comparison to the unstable acetyl-enzyme intermediate in ACh hydrolysis, the phosphorylated and carbamylated enzymes are very stable with half lives ranging from half an hour to several days (Main, 1976). Since the irreversible inhibitors undergo the same qualitative reaction as ACh itself (i.e. they form an acylated enzyme intermediate which is subsequently hydrolysed) they can therefore be classified as substrates which are only inhibitors by virtue of the stability of their acyl-enzyme intermediate complexes.

## 7. Distribution of pseudocholinesterase and its physiological function

In previous sections, the distribution of cholinergic synapses in both the peripheral and central nervous systems has been described. Since the physiological function(s) of pseudocholinesterases (pseudoChE's), and more specifically their role, if any, in cholinergic transmission, remains to be determined, it was considered inappropriate to discuss in detail their distribution under such headings. The present section will therefore describe the distribution of pseudoChE's in: a) peripheral non-nervous tissues; b) the peripheral nervous system and c) the central nervous system. In addition, the possible functions of this enzyme are also discussed (section d).

### a) Distribution in peripheral non-nervous tissues

PseudoChE's (BChE in man - see note on nomenclature, section 5) can be found in almost all the major systems of the mammalian body, but there are considerable species differences in the levels of activity and the precise distribution of these activities. It should therefore be borne in mind that relatively little work has been carried out on the



distribution of BChE in man and that the following discussion applies largely to observations made in other species. Since the subject has been thoroughly reviewed elsewhere (Silver, 1974) only a brief account will be given here.

The most extensively studied source of peripheral pseudoChE's is the plasma, where their role in anaesthetic metabolism and involvement in, for example, suxamethonium apnoea, have resulted in this enzyme being the subject of numerous studies. PseudoChE's are also found in the vascular system where they occur in the heart and the endothelium and smooth muscles of the blood vessels. PseudoChE's have also been demonstrated in association with the smooth muscle of the bronchi and bronchioles in the respiratory system. The digestive system and associated structures including the salivary glands, alimentary tract, liver and pancreas, also contain species-specific distributions of pseudoChE, as do structures of the urogenital system including the kidney and bladder (Silver, 1974).

#### b) Distribution in the peripheral nervous system

Probably the most extensively studied pseudoChE-containing structure of peripheral nervous system is the BChE-containing superior cervical ganglion of the cat. Early light microscopic studies suggested that the BChE was not associated with the neuronal cell bodies and it was concluded that the enzyme was associated with the Schwann cells (Koelle, 1951). However, more detailed electron microscopic studies have shown that the majority of the BChE is associated with the dendritic and the perikaryal (cell body) membranes of the ganglion cell (Davis and Koelle, 1978, 1981).

PseudoChE's have also been reported to occur at the neuromuscular junction of chickens (Lyles et al, 1979, 1980, 1982; Silman et al, 1979) rats (Vigny et al, 1978b) and mice (Sung, 1982) and BChE has been reported to be the predominant form of cholinesterase (73 to 85%) found at the end-plates of rhesus monkeys (see Main, 1976).

#### c) Distribution in the central nervous system

At one time it was thought that the central nervous system contained only AChE (see Silver, 1974). It was subsequently demonstrated, however, that pseudoChE was present in appreciable amounts not only in dogs (Burgen and Chipman, 1951) but also a number of other species, including man (Ord and Thompson, 1952). In the human CNS subcortical white matter has higher BChE activities than cortical grey matter (Ord and Thompson,



1952; Foldes et al, 1962; Robinson, 1966) although other areas of grey matter for example cerebellar cortex, thalamus, globus pallidus and caudate nucleus have higher activities than the white matter (Ord and Thompson, 1952; Foldes et al, 1962; Friede, 1967).

The pseudoChE of white matter has been shown to be associated with astrocytes in the brains of the rat, cat and chicken (see Silver, 1974). However, Friede (1967) reported that the BChE activity in white matter of various species, including man, was associated not with astroglia, but with the oligodendroglia. Furthermore, in the human and cat cortical grey matter, it has been shown that BChE activity was associated with neuronal cell bodies rather than glial cells (Robinson, 1966; Roessmann and Friede, 1966; Friede, 1967). The presence of BChE associated with neuronal cell bodies has also been suggested to occur in the rat brain (Shute and Lewis, 1963) and both BChE and AChE activities have been demonstrated in isolated neurons from rat cortex (Ochea et al, 1982). More specific analysis of individual neurons from the rat and human substantia nigra have demonstrated BChE and AChE activity associated with the same, but not all, neurons (Pavlin, 1983). Recently, Graybiel and Ragsdale (1982) have described a striking compartmentalization of BChE - which does not parallel AChE - in functional subdivisions of the visual thalamus and cortex of the macaque monkey, and this in addition to the observations that pseudoChE's are associated - at least in certain areas - with neuronal cell bodies, suggest that this enzyme may be involved in certain aspects of neuronal function.

In conclusion, it would seem that pseudoChE's found in the central nervous system are associated with both glial cells and neurons. Furthermore they are found in the periphery associated with sympathetic ganglia, the neuromuscular junction, in smooth muscle and also in large amounts in plasma. The function of these enzymes associated with various neuronal and non-neuronal structures remains to be determined and various suggested functions are discussed below.

#### d) Physiological functions of cholinesterases.

The misleading assumption, made in the early days of cholinesterase research, that the CNS contained only AChE (see Silver, 1974) lead to the assumption that pseudoChE played no role in neural transmission. This concept had become well entrenched by the time that pseudoChE was demonstrated in the CNS. The observations that within regions of grey matter, BChE appears to be associated, at least in part, with nerve cell



bodies both in humans and other species in addition to its localization at the neuromuscular junction and sympathetic ganglia (see above) along with the fact that brain potentials appear to be affected by the activity of BChE (Desmedt and La Grutta, 1955) are indicative of a possible role for this enzyme in neurotransmission. In addition, Graybiel and Ragsdale (1982) suggested, on the basis of the independent distributions of BChE and AChE in the monkey visual systems and their different responses after removal of an eye, that "BChE or its endogenous substrate may be a neuroactive substance in the primate brain".

The work of Koelle et al, (1976, 1977a,b), who measured the recovery of total AChE and BChE in sarin-poisoned cat superior cervical ganglia, was interpreted to suggest that BChE might be a precursor of AChE, particularly since both enzymes have a similar postsynaptic localization (Davis and Koelle, 1978, 1981). However neither cat muscle nor rat ganglia (Koelle et al, 1977a, 1979) showed compatible results. In addition, in rats, pre-and post-natal injections of a BChE inhibitor had no effect on the development of the animal and particularly AChE at the muscle end-plate (Brzin et al, 1980). Furthermore, the thermal sensitivity and immunoreactivity of the two enzymes are different (section 9). Whilst these dissimilarities would suggest that BChE is unlikely to be a precursor of AChE, the data of Koelle et al (1976, 1977a,b) are nevertheless compatible with some sort of joint regulatory mechanism for AChE and BChE. However, the two enzymes in various rat tissues, including the superior cervical ganglion have been shown to respond differently to hereditary (Edwards and Brimijoin, 1982) and hormonal factors (Edwards and Brimijoin, 1983a). More specific analysis of the individual molecular forms (section 9) has shown that they appear to be controlled in parallel in chicken muscle (Lyles et al, 1979; Silman et al, 1979) although a later report suggested that this control was not as close as originally thought (Lyles et al, 1982). In contrast, in the rat superior cervical ganglion the molecular forms of AChE and BChE appear to be controlled independently of each other (Klinar et al, 1983).

Recently, BChE has been shown to have both amidase and peptidase activities towards substance P (Lockridge, 1982) but not peptidase activity towards leu- or met-enkephalin (Chubb et al, 1982). However, the low turnover number of the enzyme towards substance P was interpreted by Lockridge as indicating that substance P behaved like an irreversible inhibitor.

Interestingly, BChE has an active site structure (section 6) similar



to the serine proteases (for example trypsin, chymotrypsin and elastase) which, like BChE, contain an active site serine that is activated by the presence of an adjacent imidazole group of a histidine residue (Stryer, 1975). The mechanism of action of serine proteases are also comparable with that observed for the choline-ester hydrolysis by BChE. Thus, the activated serine of the serine protease attacks the carbon atom of the peptide bond carbonyl group and is therefore analogous to the activated BChE serine attacking the carbon of the choline-ester carbonyl group. Consequently, it may be possible that BChE has a proteolytic function and the choline-ester hydrolytic properties of this enzyme may merely be a consequence of the active site structure and be unrelated to the physiological functions of this enzyme.

#### 8. Physiological functions of acetylcholinesterase

In contrast to BChE, a physiological function of AChE, namely the hydrolysis of ACh, has long been established (Silver, 1974; Main, 1976). However, various other functions of AChE have been proposed and will be described in the present section.

The occurrence of AChE outside the nervous system in diverse structures such as the erythrocyte membrane, vascular tissue and the placenta has lead to suggestions that AChE may be involved in the regulation of permeability at these sites although the data remains unconvulsive (for fuller discussion see Silver, 1974). Furthermore, in relation to cholinergic neurotransmission, many years ago it was hypothesised that AChE and the ACh receptor may be the same molecule. However, the evidence for this hypothesis has also been reviewed by Silver (1974) and no longer seems tenable. Nevertheless, a recent report has suggested that the postsynaptic receptor - although not the same molecule as AChE - may be regulated in some unknown manner by AChE (Fossier et al, 1983).

With respect to a role of AChE in non-cholinergic neuronal mechanisms, it is interesting to note that the majority of central of cholinergic synapses are muscarinic in nature and are therefore characterised by a slow and delayed response. Consequently, the requirement for rapid hydrolysis of ACh at such synapses is presumably not as crucial as at synapses characterised by a fast nicotinic-type response. Therefore, at such synapses it may be possible that AChE, in

addition to hydrolysing ACh, may also perform a second function. A similar deduction was made by McLennan (1970) using a different argument, when he stated that "it is becoming increasingly clear that the action of anticholinesterase drugs, while striking at the neuromuscular junction, is rather slight upon responses elicited by acetylcholine within many parts of the central nervous system, which again suggests that the enzyme is not primarily responsible for termination of the acetylcholine effect".

A possible function for AChE unrelated to the termination of ACh has recently been suggested by the observation that exogenous AChE injected into the substantia nigra depressed the rate of firing of cells within this region and produced marked behavioural responses. This action was shown to be unrelated to the hydrolysis of ACh since injections of BChE with an ACh-hydrolytic capacity approximately ten times that of the injected AChE produced no such effect (Greenfield et al, 1981, 1984). Furthermore, infusions of amphetamine into the rabbit, which stimulates the release of dopamine, also increased the release of AChE, the conclusion being that AChE release and dopamine neurotransmission may be inter-related (Greenfield and Shaw, 1982), possibly due to a modification of the striatal dopamine receptors by AChE (Greenfield et al, 1984).

In addition, it has been demonstrated that AChE possesses non-ACh hydrolytic properties. Thus, AChE demonstrated amidase properties towards the amide analogue (Moore and Hess, 1975) and peptidase activity towards substance P (Chubb et al, 1980) and towards leu- and met-enkephalin (Chubb et al, 1982) and it has been suggested that AChE may play a role in the intracellular post-transcriptional processing of peptide precursors (Chubb and Millar, 1984).

A phenomenon of AChE that has been observed in both in vivo and in vitro studies and might prove to be of great functional importance is the observation that AChE is secreted from both muscle and nervous tissues. In this respect, cultured chick muscle cells have been extensively investigated, and it has been shown that these cells release AChE into the medium (Wilson and Walker, 1974; Rotundo and Fambrough, 1979) and more recently cultured quail muscle cells have also been reported to secrete AChE (Bulger et al, 1982; Wilson and Nieberg, 1983). Furthermore, this is a genuine secretory phenomenon, and not merely a sloughing off of membrane-bound enzyme (Smilowitz, 1980), and the antibiotic tunicamycin, which interferes with the glycosylation of proteins, blocks secretion (Rotundo and Fambrough, 1980a,b). Not only is



AChE released from cultured muscle cells but the muscle itself also continually releases AChE and the major secreted molecular form (see section 9 for description of molecular form nomenclature) is the  $G_4$  molecule (Carter and Brimijoin, 1981). In addition, AChE is also released from cultured embryonic chick spinal cord, neuroblastoma and brain neurons (Oh et al, 1977). More specifically when AChE molecular forms are examined it is found that mouse neuroblastoma cells release all three globular forms i.e.  $G_1$ ,  $G_2$  and  $G_4$ , with the  $G_4$  being the major secreted form (Lazar and Vigny, 1980; Kimhi et al, 1980).

In the peripheral nervous system AChE has also been shown to be released in vivo from bovine adrenal glands, derived at least partly from the splanchnic nerves and subsequently shown to be the  $G_4$  form (reviewed by Chubb and Hodgson, 1982), whilst the  $G_4$  form has been reported to be specifically released from the rat superior cervical ganglion (Gisiger and Vigny, 1977). AChE has also been shown to be released from dog sciatic nerve axons (see Chubb and Hodgson, 1982) and from the nerve terminal of the neuromuscular junction in response to nerve stimulation where analysis of molecular forms showed that the  $G_4$  form was the major secreted species (Skau and Brimijoin, 1978).

Whilst the demonstration of in vivo AChE release within the CNS is much more difficult, it has however been shown, histochemically, that AChE is released from motor neuron perikarya (Kreutzberg and Toth, 1974; Kreutzberg et al, 1975). Release of AChE from areas of the brain itself have been suggested by the observations of elevated AChE in the CSF of rabbits and cats following stimulation of the central end of the cut sciatic nerve and clamping of the rabbit foot (see Chubb and Hodgson, 1982). In addition direct electrical stimulation or cooling of the substantia nigra and caudate nucleus as well as pharmacological administration of chlorpromazine elevates AChE in rabbit CSF (see Chubb and Hodgson, 1982). By using surgically implanted push-pull cannulae it has been possible to demonstrate that AChE is spontaneously released from both the substantia nigra and caudate nucleus of rabbits and cats, whilst BChE was released from both structures in the cat but only from the substantia nigra of the rabbit (Greenfield et al, 1980; Greenfield and Shaw, 1982). In addition, whilst AChE-release was affected by application of high potassium concentrations in the cat and by amphetamine in rabbits, BChE-release remained unaltered (Greenfield et al, 1980; Greenfield and Shaw, 1982).

Despite the fact that AChE release is now a well established

phenomenon, both in the peripheral and central nervous systems, the physiological functions served by such a mechanism are unknown (Chubb and Hodgson, 1982). It may, however, be possible that in cholinergic structures, the AChE could be stored in the presynaptic vesicles, in which the high concentration of ACh (approximately 100mM; Barrett and Magleby, 1976) would inhibit the enzyme hydrolytic activity, and that when discharged, the enzyme might diffuse across the synapse and interact with the postsynaptic receptor (Fossier et al, 1983). Alternatively, and assuming a role for AChE in the post-transcriptional modification of peptide precursors, the secretion of AChE may merely be a consequence of the enzyme being located in the same secretory granules as the peptide substrate (Chubb and Millar, 1984).

### 9. Molecular forms of cholinesterases

Since the electric organs of both the Torpedo (marine electric ray) and Electrophorus (electric eel) correspond to phylogenetically modified muscles and as such possess a high density of cholinergic synapses and consequently exceptionally high concentrations of AChE, they have been extensively used in studies elucidating the quaternary structure of AChE since these organs. The occurrence of discrete molecular forms of both AChE and pseudoChE that differ with respect to their quaternary structure is now well established and in the following discussion some of the principle features of the quaternary structure of electric organ AChE will be described and will be related to AChE and BChE derived from other sources. A detailed and comprehensive review has recently been published on this subject (Massoulie and Bon, 1982).

Massoulie and co-workers (Massoulie and Rieger, 1969; Massoulie et al, 1970a,b; Bon et al, 1973) found that the AChE present in homogenates of Electrophorus electric organ prepared in the presence of high concentrations (1M NaCl) could be separated according to their size and shape into three distinct forms using ultracentrifugation and gel filtration chromatography. Electron microscopy revealed that these three forms were highly asymmetric molecules consisting of a globular "head" associated with a rod-shaped tail approximately 50nm long (Dudai et al, 1973; Rieger et al, 1973). These three Electrophorus forms were subsequently shown to contain one, two or three tetrameric groups of subunits (Bon et al, 1976), corresponding to 4, 8 and 12 active sites



respectively (Vigny et al, 1978a) and each subunit had a molecular weight of approximately 80,000 (Rosenberry and Richardson, 1977). The "tail" had a molecular weight of approximately 100,000 (Bon et al, 1976) and was collagen-like since not only were hydroxyproline and hydroxylysine residues - which are characteristic of collagen - present (Rosenberry and Richardson, 1977; Anglister and Silman, 1978) but it was also sensitive to collagenase (Johnson et al, 1977; Anglister and Silman, 1978). In addition, the tail had a characteristic collagen-like triple helical structure as revealed by the analyses of circular dichroic spectra (Rosenberry et al, 1980). The low salt aggregation properties of Electrophorus AChE seem to be a function of the collagen-like tail since molecules treated with collagenase lose this property (Bon and Massoulie, 1978), and consequently the solubilization of different molecular forms of AChE is best achieved in a high-salt, detergent-containing medium (Dudai and Silman, 1974), although it has recently been suggested that the presence of EDTA in addition to the high-salt concentration is required to extract all the asymmetric forms (Gomez-Barriocanal et al, 1981; Barat et al, 1984).

Of additional interest is the observation that proteolytic digestion converted the tailed forms to tail-less (globular) tetramers, which in turn could be converted into active dimers and monomers (Massoulie and Rieger, 1969; Massoulie et al, 1970a,b; Bon and Massoulie, 1976). Thus, Electrophorus AChE can exist as six distinct molecular forms which using the nomenclature of Bon et al (1979) can be divided into two classes: "asymmetric" forms, designated  $A_n$ , which are assemblies of  $n$  subunits attached to a collagen-like tail, and "globular" forms,  $G_n$ , in which assemblies of  $n$  subunits are devoid of any detectable collagen-like component. The structures of these six possible molecular forms are shown in figure 8.

Although the subunits may differ because of their insertion in different quaternary structures, they remain essentially identical with respect to inhibition by excess substrate, their relative rates of hydrolysis of various substrates and the turnover number per active site (Bon and Massoulie, 1976; Vigny et al, 1978a). A series of six different forms of AChE having comparable quaternary structures to those found in electric organs (i.e.  $A_{12}$ ,  $A_8$ ,  $A_4$ ,  $G_4$ ,  $G_2$  and  $G_1$ ) have also been described in bovine superior cervical ganglia (Bon et al, 1979), chicken muscle (Allemand et al, 1981), rat muscle (Bon et al, 1979; Senni et al, 1981) and human muscle (Carson et al, 1979). It therefore seems that

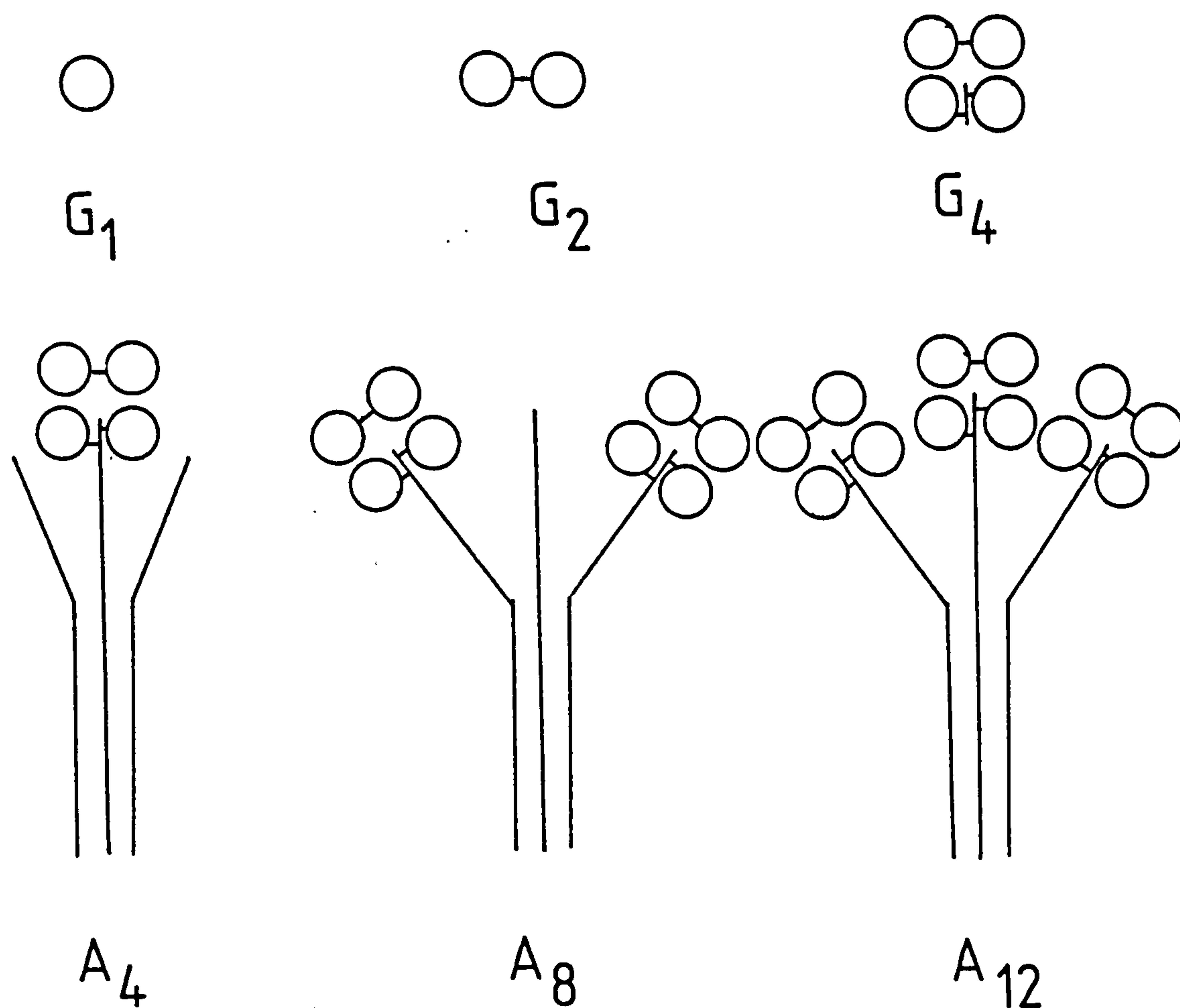


Figure 8. Schematic diagram illustrating the six possible quaternary structures of AChE (Massoulie and Bon, 1982). Molecular forms of pseudoChE have been less well characterized although it is thought that this enzyme exists as a series of molecules with quaternary structures analogous to those found for AChE (see text).

molecular forms of AChE, found in a wide variety of species, share a common series of quaternary structure. However, the presence and abundance of these different forms varies between different tissues in the same species and the same tissue of different species and is thought to reflect the physiological function of any given tissue (reviewed by Massoulie and Bon, 1982).

Since AChE is available in large quantities from electric organs, and since AChE is often considered to be more functionally important than pseudoChE, most of the work on elucidating the quaternary structure of cholinesterases has concentrated on AChE rather than pseudoChE. However, recent studies have shown that multiple forms of pseudoChE that parallel those of AChE, occur in various tissues of rat (Vigny et al, 1978b; Klinar et al, 1983) and chicken (Lyles et al, 1979, 1980, 1982; Silman et al, 1979; Allemand et al, 1981). Moreover, each AChE form appears to



possess a pseudoChE counterpart which sediments in rats slightly faster, but in chickens slightly slower, than its AChE counterpart. The solubility characteristics of the different BChE and AChE forms are also very similar (Vigny et al, 1978b; Allemand et al, 1981). In addition, the higher molecular weight forms of BChE are sensitive to collagenase indicating that, like the corresponding high molecular weight forms of AChE, they possess a collagen-like tail (Allemand et al, 1981).

The homology between pseudoChE and AChE forms is further demonstrated by the sensitivity of BChE to trypsin which, as with AChE, converts the heavy forms to lighter forms (Allemand et al, 1981). To date only one study has been carried out on the characterization of the quaternary structure of BChE, and this suggests that the structural homology between BChE and AChE indicated by their similar solubility and protease-sensitivity characteristics, is valid. Thus, Lockridge (1979) found that human plasma BChE consists of a pair of dimers that constitute a globular, tetrameric structure similar to that hypothesised for the corresponding AChE tetramer. However the two proteins are different in their sensitivity to thermal inactivation, as measured on total AChE and BChE activities (Vigny et al, 1978b) or more specifically when the molecular forms of AChE and BChE with similar quaternary structures are compared (Edwards and Brimijoin, 1983b). Furthermore AChE and BChE have been shown to differ with respect to their immunoreactivity (Vigny et al, 1978b).

It is important point to establish whether or not the in vitro molecular forms are representative of those found in vivo. It may, for example, be possible that the extraction process produces artefactual aggregation or disaggregation of the in vivo molecular forms. Lazar and Vigny (1980) studied the molecular forms of AChE in murine nerve cell cultures (T28) after solubilization in the presence of 1% Triton X-100 detergent. Prior to extraction, exposure of cells to the nonpenetrating inhibitor echothiopate selectively inhibited the  $G_4$  form leaving the  $G_1$  form essentially unaffected, thus showing that  $G_4$  and  $G_1$  forms represented discrete in vivo pools which were probably intracellular and extracellular respectively. Using a similar technique but a different nonpenetrating inhibitor, namely BW284c51, it has also been shown that a discrete in vivo pool of AChE activity exists in cultured rat pheochromocytoma PC12 cells (Inestrosa et al, 1981), murine T28 cells and chicken sympathetic neurons (Taylor et al, 1981). Again the  $G_4$  form was mainly located on the external facing surface of the cell membrane and



the lower molecular weight forms ( $G_1$  plus  $G_2$  in the rat pheochromocytoma cells,  $G_1$  in the mouse T28 cells and  $G_2$  in the chicken sympathetic neurons) located intracellularly and it is therefore likely that solubilized molecular forms do reflect the state of the enzymes in situ (Massoulie and Bon, 1982).

The metabolic relationships between the different molecular forms of AChE have been analysed mainly by observing the recovery of AChE after irreversible inactivation by phosphorylating inhibitors. Using such techniques it has been observed that in DFP-inhibited neuroblastoma cells,  $G_1$  reappears before  $G_4$  (Rieger et al, 1976) consistent with  $G_1$  being a precursor of  $G_4$ . Similarly, low molecular weight forms of AChE also recover before the higher molecular weight forms in organophosphate-inhibited cultured embryonic rat muscle cells (Koenig and Vigny, 1978) rat diaphragm (Grubic et al, 1981), rat brain (Huther et al, 1978) rat superior cervical ganglia (Gisiger and Vigny, 1977) and quail muscle cells (Wilson and Nieberg, 1983). Rather than studying the recovery of molecular forms of AChE after organophosphate-inhibition, Vimard et al (1976) examined the relationship of the molecular forms of AChE in a murine neuroblastoma cell line by blocking protein synthesis. They observed an increase in  $G_4$  form and a parallel decrease in  $G_1$  form, again indicating that  $G_4$  is assembled from  $G_1$ . Cells therefore appear to progressively incorporate the catalytic subunits from intracellular forms, probably associated with the endoplasmic reticulum, into a plasma membrane  $G_4$  form, and in some cases into collagen-tailed forms, by active metabolic processes (Massoulie and Bon, 1982).

Despite numerous descriptions of the molecular forms of AChE and to a lesser extent pseudoChE, relatively little is known about the physiological functions of the individual forms, although much attention has been focussed on the  $A_{12}$  form of AChE, which was initially thought to be specific to muscle motor end-plates. Thus, initial reports suggested that this form appeared to be particularly associated with end-plate regions of rat muscle (Hall, 1973; Vigny et al, 1976a,b; Bon et al, 1979; Fernandez et al, 1979) and that denervation dramatically reduced the amount of this form in rat (Hall et al, 1973; Vigny et al, 1976b; Fernandez et al, 1979; Carter and Brimijoin, 1981; Senni et al, 1981) and chicken (Vigny et al, 1976a; Sketelj et al, 1978) muscle whilst reinnervation re-established the  $A_{12}$  form in rat muscle (Vigny et al, 1976b). Furthermore embryonic rat and chicken muscle cell cultures taken at a stage in development before neuromuscular contacts were present,



contained no  $A_{12}$  form yet this form could be induced when spinal cord neurons were added to the culture (Koenig and Vigny, 1978; Rubin et al, 1980). However, despite initial reports that the  $A_{12}$  form was not found in smooth muscle (Vigny et al, 1976b) it has subsequently been detected in rat and chicken heart (Skau and Brimijoin, 1980; Massoulie and Bon, 1982) and it has also been described in endplate-free sections of human (Carson et al, 1979) and mouse (Rieger et al, 1984) skeletal muscles and also in the central nervous system (chapter 3, section IV.2.b). In addition, the  $A_{12}$  form of AChE has been described in embryonic non-innervated quail (Wilson and Nieberg, 1983) and mouse (Sugiyama, 1977) muscle cell cultures. Also, Rieger et al (1983) have recently reported that denervation of mouse skeletal muscle, in contrast to rat muscle, resulted in a pronounced loss of the  $G_4$  form of AChE with only a slight decrease in the  $A_{12}$  form. It would therefore appear that although the  $A_{12}$  form may be specifically localized at the endplate region of adult rat muscles and its specific appearance on formation of nerve-muscle contacts indicates an important functional role, it does not appear to be as functionally specific in other species. The functional importance of this and other molecular forms in other tissues have been less extensively studied and remain unknown.

In summary, AChE and pseudoChE's occur as parallel series of molecular forms with similar quaternary aggregated structures. Extensive studies have revealed two classes of molecular structures, asymmetric and globular, which are distinguished by the presence or absence respectively of a collagen-like tail. The polymeric forms are assembled sequentially from single, catalytically-active subunits. Numerous studies have established that the quantity and distribution of the different molecular forms of AChE and pseudoChE's varies between organs of the same species and between the same organ of different species. However, the physiological functions of the different molecular forms remain to be elucidated.

Chapter 2:

LAMINAR DISTRIBUTION OF CHOLINESTERASES AND CHOLINE ACETYLTRANSFERASE  
IN THE TEMPORAL CORTEX OF NORMAL AND ALZHEIMER-TYPE POSTMORTEM BRAIN



## I. INTRODUCTION

In order to relate the cortical cholinergic deficit that occurs in senile dementia of Alzheimer type (SDAT) to discrete functional and anatomical features of the cortex, the distribution of both acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) were measured throughout the depth of the cortex using a microsampling technique. In addition, the activity of choline acetyltransferase (ChAT) was also measured to allow the normal and pathological distribution of cholinesterases to be correlated with cholinergic function. The results obtained are discussed in relation to the distribution of pathological changes that occur within the cortex in SDAT.

Since the results of this present section will be described in relation to the six-layered lamination of the cortex, the following description will outline the major structural aspects of this arrangement of cortical nerve cells (for more detailed description see Brodal, 1969).

Thus, the neocortex can be made to fit a general scheme of a six-layered structure which becomes evident when stains for nerve cells are employed (figure 9) and these six layers are characterised by the following features:

I. The molecular, plexiform or zonal layer which lies immediately beneath the pia is rich in fibres - which form a dense tangentially-running plexus - but poor in neurons.

II. The external granular layer comprises densely packed small cells, some of which are pyramidal, others round or star shaped.

III. The external pyramidal layer consists predominantly of medium sized pyramidal cells, which increase in size from outside inwards. Layers II and III contain relatively few fibres.

IV. The internal granular layer is dominated by small cells lying close together, which are mostly star-shaped, although some are of the pyramidal type. This layer contains abundant horizontal fibres which form the outer band of Baillarger.

V. The internal pyramidal or ganglionic layer, comprising mainly pyramidal cells which are mostly medium and large sized. As with the pyramidal cells in layer III, they have long apical dendrites directed towards the molecular layer, and a generous supply of horizontally running basal dendrites. Horizontal fibres in this layer form the inner band of Baillarger.

VI. The multiform or fusiform layer contains predominantly spindle shaped cells. It is frequently divided into an outer part VIa and an inner part VIb which gradually fuses with the white matter.

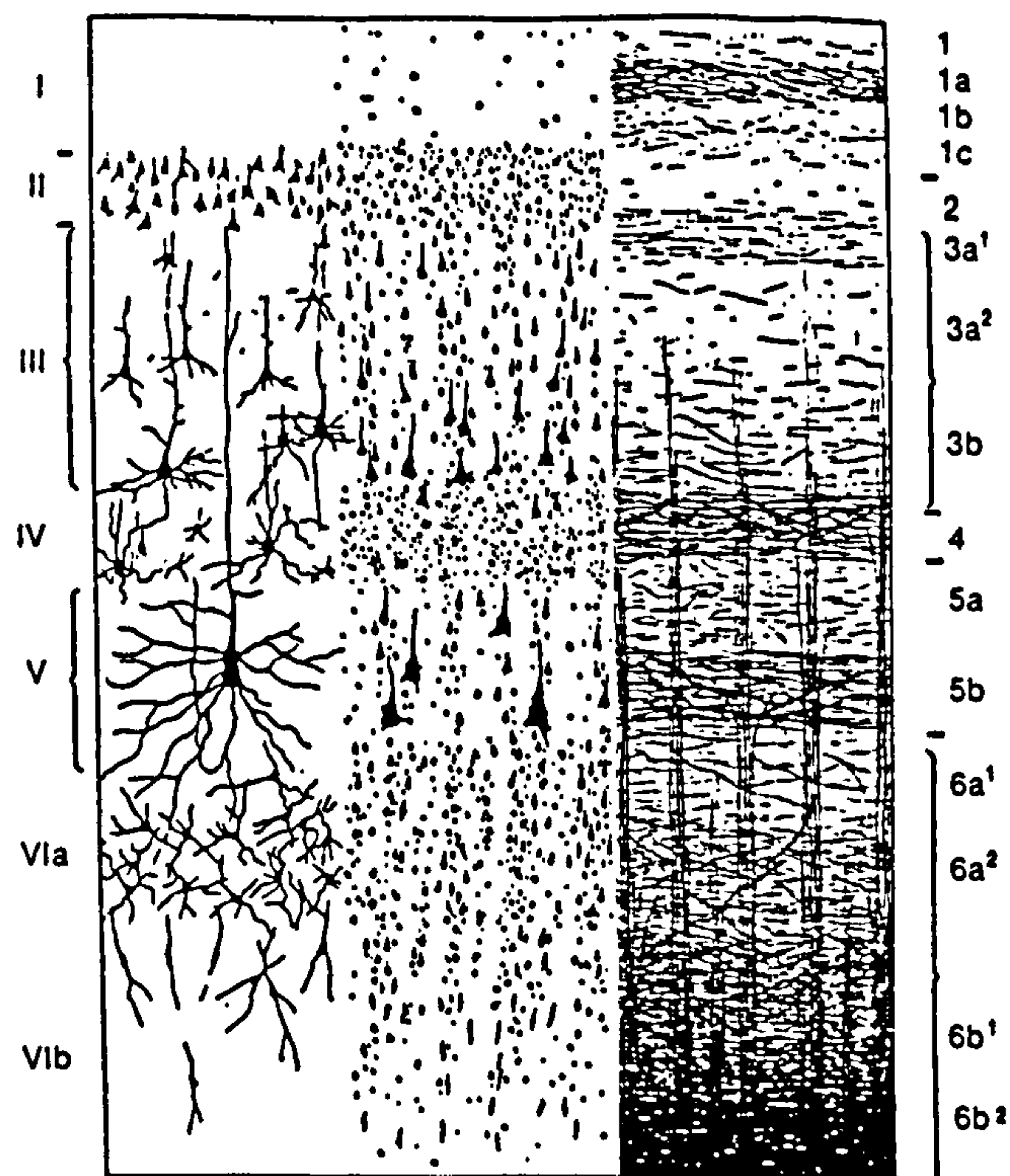


Figure 9. Diagram illustrating the layers of cells and fibres in the grey matter of human cerebral cortex. To the left staining using the Golgi method showing cell bodies and processes; centre Nissl staining showing cell bodies; right Weigert myelin stain showing fibres (Brodal, 1969).

Although the functional significance of the different cortical layers is poorly understood, the observation that areas receiving large amounts of sensory input have well developed granular layers whereas motor cortex has well developed pyramidal layers has led to the general idea that the upper cortical layers (I to IV) have predominantly receptive and associative functions, whereas the effector functions are taken care of by the deeper layers (V and VI; Brodal, 1969).

The pathological involvement of the different cortical layers and their associated neurochemical elements are of great interest in elucidating both normal and pathological aspects of cortical function. This is particularly true for SDAT which is characterised by both neuropathological and neurochemical changes within the cortex. Thus, the characteristic neuropathological features i.e. the presence of large numbers of neurofibrillary tangles and senile plaques, are not only found throughout all four lobes of the neocortex and in the phylogenetically older allocortex of the hippocampus (chapter 1, section I.6.b) but the intimate involvement of both the neocortex and hippocampus in the disease process is suggested by the correlation between the numbers of neurofibrillary tangles and senile plaques found in these areas with the



clinical severity of the disorder. (Blessed et al, 1968; Farmer et al, 1976; Wilcock and Esiri, 1982).

In addition, the major biochemical abnormality discovered to date SDAT (i.e. loss of cholinergic activity) is found in both the neocortex and the hippocampus (chapter 1, section I.7.a.i.) and the loss of cholinergic activity in these regions correlates with both the clinical and neuropathological severity of the disorder (Bowen et al, 1976; Perry et al, 1978b, 1981a; Wilcock et al, 1982; Mountjoy et al, 1984). Since the neocortex is highly organised, both structurally and functionally, it is not unreasonable to assume that there is a discrete organisation of neurochemical elements (the so-called chemoarchitecture) and that there may be a preferential pathological involvement of certain structures. However, the majority of the work carried out on neocortical cholinergic enzyme activities in SDAT has been performed on homogenates or prisms of grey matter and although such measurements provide an estimate of the overall activity within the grey matter, they cannot differentiate between any intracortical cholinergic-specific regions, which may show selective pathological involvement.

The study of the intracortical distribution of neurochemical markers in human neocortex was pioneered by Alfred Pope, who initially analysed the intralaminar distribution of AChE in biopsy specimens of frontal cortex (Brodmann area 9) from psychotic and non-psychotic patients (Pope et al, 1952). These studies were then extended to compare the intralaminar distributions of AChE in postmortem frontal cortex (Brodmann area 9) of cases of presenile dementia of Alzheimer-type (Pope et al, 1964, 1965). In these reports AChE was shown to be reduced in the cortex of demented subjects, particularly in layers II to V, and as such represent the first observations of a reduction in cortical cholinergic activity associated with Alzheimer-type dementia.

The present investigation was therefore undertaken with the aim of describing the distribution of both AChE and BChE in normal postmortem temporal cortex (Brodmann area 21) and comparing these with the distributions in comparable cortex from established cases of SDAT. In addition the cholinergic marker enzyme ChAT was also measured as a specific measure of cholinergic function. In comparison to the studies of Pope and colleagues, the present study differs in that not only are the subjects used here older but also the type of cortex analysed is different. The observed normal and pathological distribution of enzymes will be discussed in relation to the structural, functional and

pathological features of the different cortical layers. The results presented here have been published elsewhere (Perry et al, 1984a).



## II. MATERIALS AND METHODS

### 1. Cases

Cases were selected according to clinical and pathological criteria outlined in more detail elsewhere (Roth, 1955; Blessed et al, 1968; Tomlinson et al, 1968, 1970; Perry et al, 1978b). In brief, patients were placed into a clinical diagnostic category according to the criteria outlined by Roth (1955) and the intellectual and personality deterioration shown by the patient was quantified by measuring the patients' performance in a number of simple psychological tests of concentration, remote and recent memory and orientation (Blessed et al, 1968). In these tests a positive score was awarded for each correct item and the resulting mental test score could range between a maximum possible score of 37 to 0, which represents complete failure in all tasks. The mental test scores achieved using this method have been shown to exhibit a highly significant negative correlation with the number of neuropathological changes found in the brain at autopsy (Blessed et al, 1968).

### 2. Postmortem brain sampling

Brains were obtained at autopsy and transferred to a class II safety cabinet where all procedures involving fresh tissue were carried out. The whole brain was sectioned down the midline to separate right and left hemispheres. The right hemisphere was fixed in neutral 10% formalin for a period of not less than three months prior to neuropathological examination (following section). The left hemisphere was sliced coronally at approximately 10mm intervals, and the slices were identified at different levels along the anterior-posterior axis by the presence of distinct subcortical anatomical "landmarks". Using this procedure it was possible to reliably sample tissue from the same region of different brains.

In the present study, the mid-temporal gyrus was dissected out at the caudal putamen/mid-substantia nigra level and the attached surface membranes were removed. Columns of cortical material (2mm x 2mm cross-section x 4mm depth) were prepared from the relatively straight



edge of the superior surface of this gyrus (Brodmann area 21) as shown in figure 10.

The blocks for biochemical analyses were attached at their base to thin paper strips, running parallel with the pial surface and immediately snap-frozen and stored in liquid nitrogen. Blocks adjacent to those used for biochemistry were also mounted on paper strips, but this time perpendicular to the pial surface. These columns were then fixed in 10% neutral formalin before histological examination of the laminar structure using Nissl staining.

### 3. Neuropathological examination

After a suitable period of fixation (not less than three months), the whole right hemisphere was sliced coronally at intervals of approximately 10mm. Each slice was examined macroscopically for ischaemic lesions and their extent and position noted. A record was also made of other gross features such as gyral atrophy, ventricular enlargement and substantia nigra pigmentation.

Samples were then removed from two frontal, one temporal, one occipital and two parietal regions, along with a sample of hippocampus, for histological examination (for detailed description see Blessed et al, 1968). In brief, from each of these regions 25µm sections were cut on a freezing microtome and silver stained by von Braunnuhl's method to demonstrate senile plaques, whilst from the same regions paraffin sections were also cut (20µm) and stained using the Palmgren technique for the demonstration of neurofibrillary tangles. The numbers of senile plaques was recorded in five different 1.3mm diameter fields from each neocortical area and the mean of the number of plaques present in the 30 different microscopic fields was used as an index of the severity of the dementia (Blessed et al, 1968). In addition, the number of neurofibrillary tangles in all cortical areas was recorded on the basis of their relative abundance (0 = absent, +++ = very abundant).

In addition to sections stained for plaques and tangles, sections were also cut and stained using routine methods. Thus, 5µm paraffin sections were stained using the general histological haematoxylin and eosin stain and separate 20µm paraffin sections were stained using the cresyl fast violet and Loyez techniques. Using these procedures it was possible to routinely identify CNS disease due to factors such as



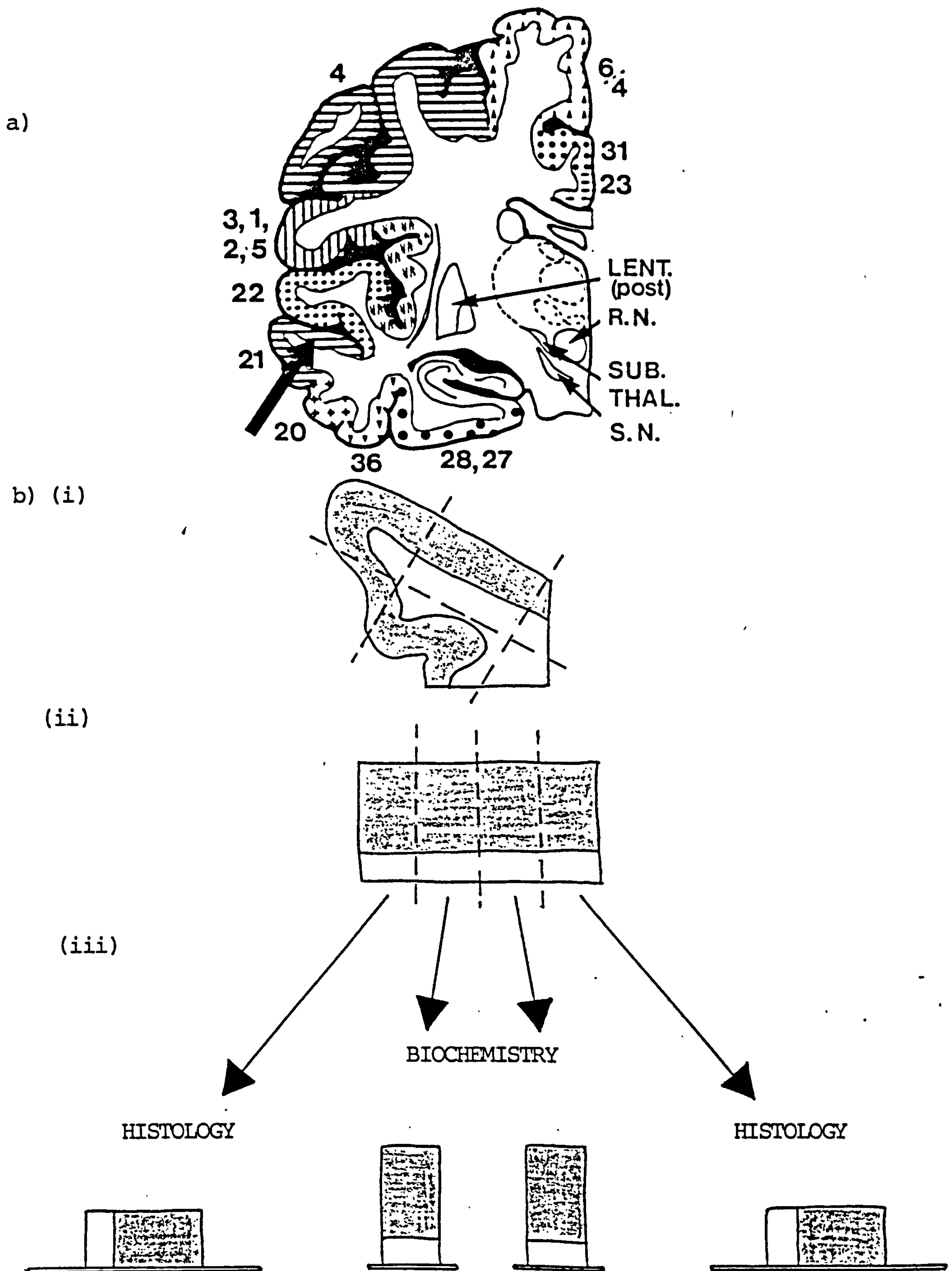


Figure 10. Diagram illustrating the sampling procedure used in the preparation of cortical columns for subsequent biochemical analyses or histological examination of cortical structure.

a) Coronal level from which tissue was removed. Subcortical anatomical "landmarks" are: LENT. (post), posterior lenticular nucleus; R.N., red nucleus; SUB. THAL., subthalamic nucleus, S.N., substantia nigra. Numbers refer to Brodmann areas of the cortex. Large arrow indicates superior surface of the mid-temporal gyrus from which tissue used in this study was removed.

b) Dissection of cortical columns for both biochemistry and histology. Dashed lines illustrate dissections.

(i) Trim grossly dissected gyrus so that the pial surface is parallel to the horizontal plane

(ii) Dissect gyrus into columns approx. 2mm x 2mm x 4mm

(iii) Mount columns onto filter paper base

hypertensive cortical changes, alcoholic brain damage, demyelinating diseases, progressive supra nuclear palsy, cerebral tumours, infective processes such as viral encephalitis, Huntington's and Pick's disease and acute or chronic traumatic damage. Such cases were excluded from the present studies.

#### 4. Sectioning of cortical columns

The frozen cortical columns were removed from the nitrogen fridge and transferred to a container of dry ice prior to cryofixing the tissue onto a microtome chuck. For this purpose it was found that a base of moist filter paper frozen to the chuck and onto which the paper strip attached to the base of the column was frozen, offered the best means of mounting the cortical column (cortical columns cryofixed directly onto the chuck tended to become detached more readily than those attached to a filter paper base). In order to prevent the tissue thawing, the mounting procedure was carried out as rapidly as possible. The chuck was then transferred to a cryostat and the cortical column was sectioned parallel to the pial surface. Serial 20 $\mu$ m sections were cut at a temperature of -9°C or -12°C depending on whether the ambient temperature was cool or warm respectively. At temperatures below these the slices shattered and became difficult to handle whereas at higher temperatures there was the risk that air currents within the room might enter the cryostat and thaw the sections as they were cut. Consecutive series of five 20 $\mu$ m slice were collected and transferred to vials pre-cooled in dry ice (five 20 $\mu$ m sections were cut rather than a single 100 $\mu$ m section because the resistance of the block to the knife was so high when cutting a 100 $\mu$ m section that the column invariably broke at the grey/white matter interface or was pulled away from its mounting).

#### 5. Chemicals

Chemicals purchased from the Sigma Chemical Company Limited (Poole, England) included: acetylthiocholine iodide, albumin (bovine serum; RIA grade), 1:5-bis (4-allyldimethylammonium phenyl) pentan-3 one dibromide (BW284c51), 5,5-dithiobis-2-nitrobenzoic acid (DTNB), Folin and Ciocalteu's phenol reagent (2 molar) and sucrose (grade I). Copper



sulphate pentahydrate (AnalaR grade), potassium sodium tartrate (AnalaR), sodium hydrogen carbonate (AnalaR), sodium hydroxide (AnalaR), sodium phosphate (monobasic and dibasic; AnalaR) and Triton X-100 (Scintran grade) were all obtained from BDH (Poole, England). Radiolabelled  $^{14}\text{C}$ -acetyl Coenzyme A ( $>50\text{mCi/mmol}$ ) was purchased from Amersham International plc (Amersham, England).

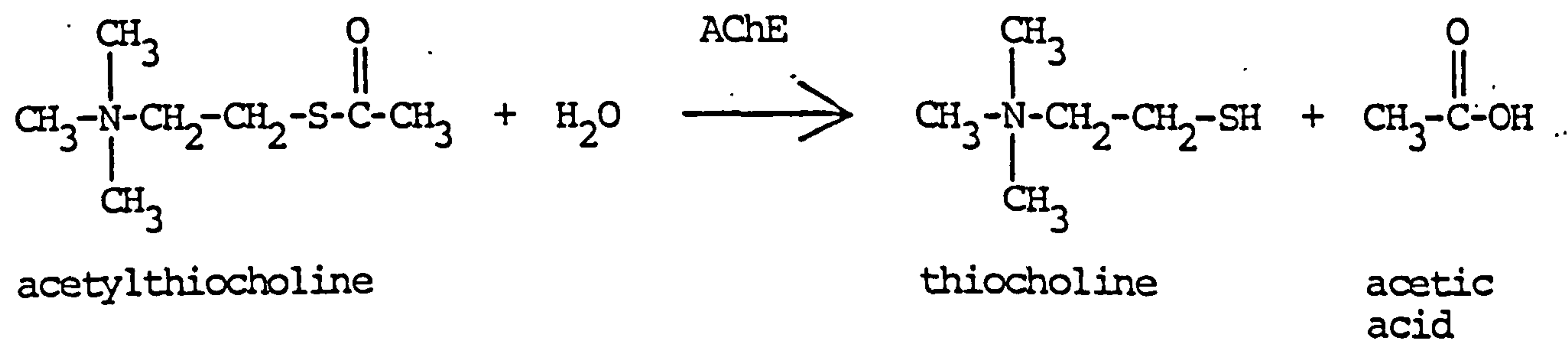
## 6. Biochemical analyses

To each collection of five slices were added 50 $\mu\text{l}$  of 0.32M sucrose containing 0.5% v/v Triton X-100 detergent. The tissue was dispersed on ice by passing the suspension 20 times through a 100 $\mu\text{l}$  fixed-needle syringe (Terumo, Tokyo, Japan).

AChE and BChE were assayed using the colorimetric method of Ellman et al (1961). The principle of this assay procedure is that thiocholine released by the enzymatic hydrolysis of the substrate (which is usually acetyl-, acetyl- $\beta$ -methyl-, or butyrylthiocholine) reacts with Ellman's colour reagent (5,5-dithiobis-2-nitrobenzoic acid; DTNB) to produce the yellow, 5-thio-2-nitrobenzoic acid anion. Since this anion has an absorbance peak at a wavelength of 412nm the rate of its production can therefore be measured spectrophotometrically. The reaction procedure is illustrated in figure 11 using acetylthiocholine as substrate.

Cholinesterase activities were measured at room temperature in 0.1M phosphate buffer, pH 8.0 using 10 $\mu\text{l}$  aliquots of the dispersed tissue in 1.0ml total incubation volume. Reactions were carried out (following a 30 minute preincubation in medium minus substrate) in the presence and absence of  $10^{-5}\text{M}$  BW284c51 - a specific AChE inhibitor (see chapter 1, section II.5) - using  $0.5 \times 10^{-3}\text{M}$  acetylthiocholine iodide as substrate and a concentration of 3.3mM DTNB (100mM DTNB stock solution was made up in 0.1M phosphate buffer, pH7.0 containing 18mM sodium bicarbonate). The activities that were sensitive and insensitive to BW284c51 were considered to be due to AChE and BChE respectively. Although the conditions were not optimal for the estimation of BChE, they provided a convenient measure of activity in large numbers of samples. ChAT was assayed using the method of Fonnum (1975), whilst protein was measured by the method of Lowry et al (1951) using bovine serum albumin to construct a calibration curve.

a) Enzymatic hydrolysis of acetylthiocholine to produce thiocholine and acetic acid



b) Reaction of free thiol group of thiocholine with DTNB to produce the yellow 5-thio-2-nitrobenoic acid anion

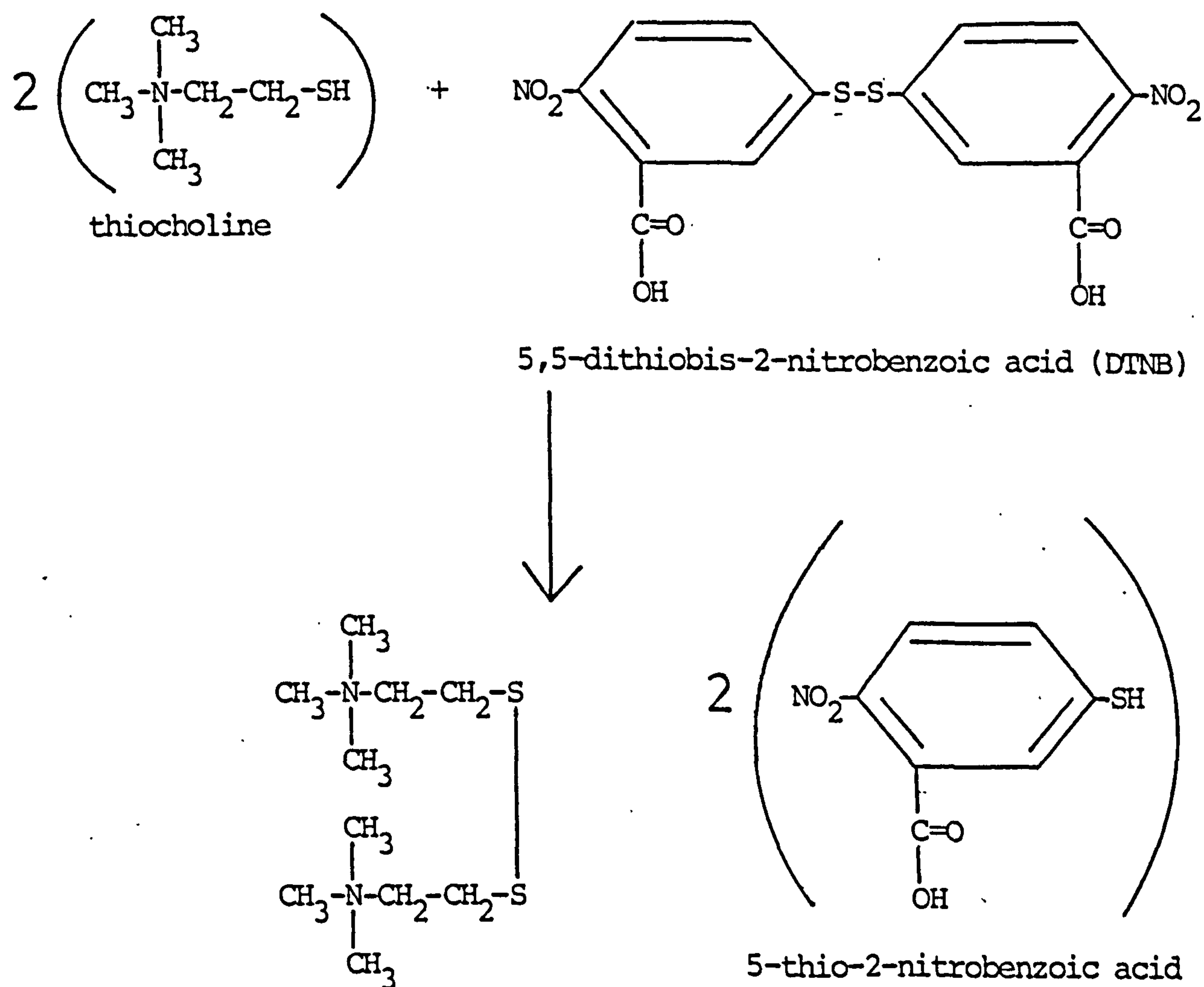


Figure 11. Illustration of the reaction scheme (shown here for the hydrolysis of acetylthiocholine by AChE) for the Ellman colorimetric assay of cholinesterases.



## 7. Statistical analysis

A more detailed description of the statistical methods used in the present experiments is available elsewhere (Perry et al, 1984a). In brief, an analysis of variance (ANOVA) was carried out in both normal and SDAT patient categories using repeated measures of the slice number (cortical level) and the enzyme activity to determine whether or not the relative levels of any given parameter varied between different cortical levels (i.e. was there a discrete laminar distribution of the enzyme activity or was the enzyme distributed evenly through the depth of the cortex). Furthermore, for each enzyme, a two factor (patient category versus slice number) analysis was performed to establish whether there was a significant difference between the distribution of that particular enzyme in the SDAT compared to normal cortex. Where a difference in overall distribution through the depth of the cortex was found to be statistically significant, the data were subjected to more detailed examination to delineate those regions where the activities in the normal and SDAT were significantly different. The approach used was the "Newman-Keuls" method (Winer, 1971) in which the means contrasted under the ANOVA terms were arranged in rank order, and the "critical difference" was calculated by the "least significant difference" method, which is equivalent to always using a weighting factor derived from  $r=2$  in the Newman-Keuls method (Winer, 1971). When this produced an apparent value of less than  $P<0.05$ , it is reported as a "strong trend".

## RESULTS

A comparison of the two groups of subjects used in the present study is shown in table 11. Both groups were well matched for age, sex, brain weight, postmortem delay and cortical grey matter thickness. The cases of SDAT were well established with a mean plaque count ranging from 41-48. In contrast, the normal group had plaque counts well within the normal range (Tomlinson et al, 1968)

Figure 12 shows a photomicrograph of a representative Nissl stained section of the tissue sampled for histology from one normal and one SDAT case, with the six layers of the cortex marked on. The Nissl stained histological sections showed that, in addition to the observation that total cortical thickness did not vary between normal and SDAT cortex, there was also no obvious difference in the relative thickness of the different layers between the groups, although the demarcation between cortical layers was not always clearcut. In the SDAT cases, senile plaques were generally most prevalent in the middle layers (III to V).

### 1. Distribution of acetylcholinesterase

The laminar distribution of AChE within the cortex of normal and SDAT cases is shown in figure 13 and analysed in table 12. The normal distribution of AChE shows peaks of activity associated with layers I/II, III/IV and VI, with greatest activity associated with the upper cortical layers, and a trend of decreasing activity as the sub-cortical white matter is approached.

In the SDAT cases substantial reductions in AChE activity were seen throughout the cortex but not the white matter. Statistically, the sum total (across slices) of AChE throughout the cortex was significantly different ( $F=4.45$ ;  $P<0.05$ ) between the normal and SDAT cases in agreement with measurements obtained using whole homogenates of grey matter. More specifically, the enzyme distribution pattern was greatly altered in SDAT, with activity no longer resolving into three distinct peaks and, unlike the normals, there was no tendency for the upper cortical layers to have highest activities. Thus, the residual enzyme was found mainly in layer III with decreasing activity either side of this region as layers I or VI were approached such that the activities found in these



TABLE 11

Comparison of the normal and SDAT cases used in the present study<sup>a</sup>

	Normal	SDAT
Age, years	78 $\pm$ 6	79 $\pm$ 6
Sex: male/female	3/2	2/3
Brain weight, g.	1242 $\pm$ 103	1186 $\pm$ 166
Postmortem delay, hours <sup>b</sup>	29 $\pm$ 9	34 $\pm$ 8
Cortical thickness, mm.	2.83 $\pm$ 0.15	2.60 $\pm$ 0.48
Mean plaque count, range <sup>c</sup>	0-6	41-48

<sup>a</sup> Values shown are mean  $\pm$  standard deviation (n=5, both groups).

<sup>b</sup> Postmortem delay is the time between patient death and sampling of the brain.

<sup>c</sup> Mean count of the number of plaques in a 1.3 mm diameter optical field of several different neocortical regions (Blessed et al, 1968).



## Mid temporal cortex

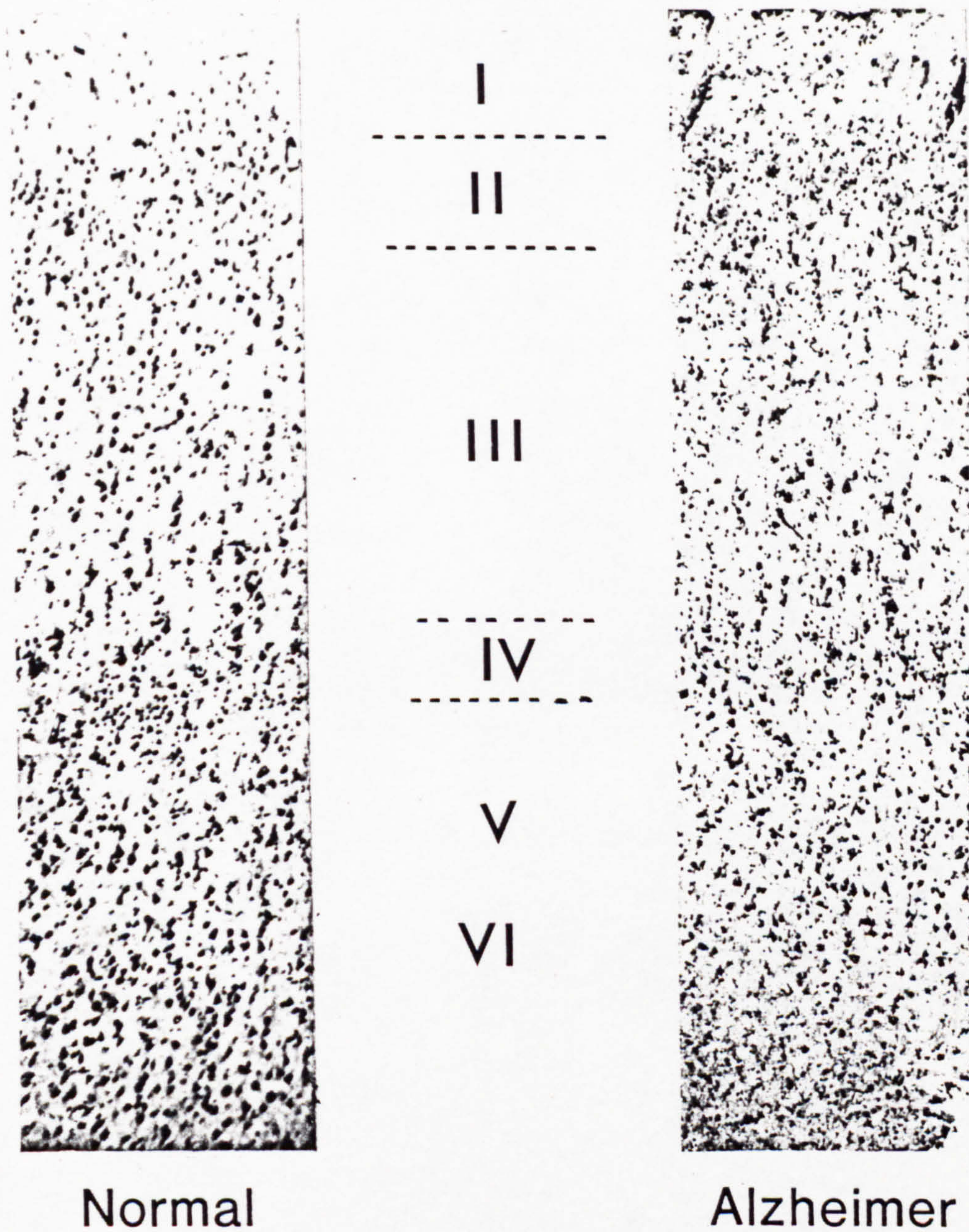


Figure 12. Photomicrographs of cryostat sections prepared from columns of temporal cortex (Brodmann area 21) immediately adjacent to those used for laminar neurochemical analyses (normal left; SDAT right). The various layers are indicated, layers V and VI being slightly contracted in this particular case of SDAT.



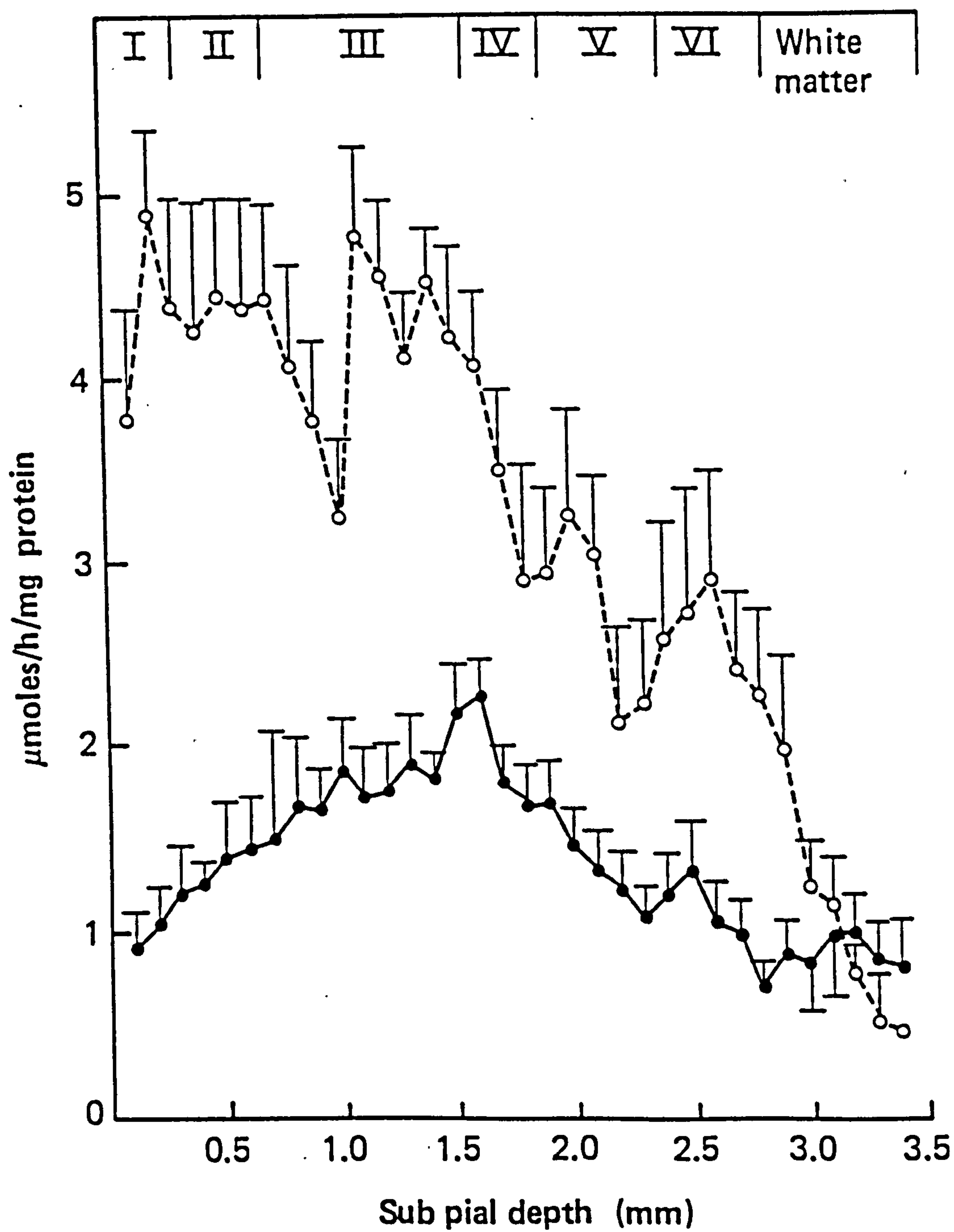


Figure 13. Laminar distribution of acetylcholinesterase activities in temporal cortex (Brodmann area 21) from normal (O---O) and SDAT (●—●) cases. Points shown are mean values ( $n=5$ , both groups) with vertical bars representing the standard errors. The average positions of the six cortical layers and white matter are also shown.

TABLE 12

Analysis of variance (ANOVA) for enzyme data

Source of variation	Degrees of freedom	Variance ratio (F)
<u>Acetylcholinesterase</u>		
A: Patient category	1,5	** 9.84
B: Slices	31,606	*** 4.95
AB interaction	31,606	*** 3.14
<u>Butyrylcholinesterase</u>		
A: Patient category	1,5	3.71
B: Slices	31,606	*** 11.05
AB interaction	31,606	1.43
<u>Choline acetyltransferase</u>		
A: Patient category	1,5	*** 18.11
B: Slices	31,606	*** 7.71
AB interaction	31,606	*** 4.69

\*\*, \*\*\* Statistically significant: P < 0.01 and 0.001 respectively



two layers were comparable to those found in the white matter. Statistical treatment of these results showed that the distribution of enzyme in SDAT was significantly altered at several points ( $P < 0.05$ ; "least significant difference" method) with greatest divergence observed in the outer cortical layers I, II and the outer part of layer III.

## 2. Distribution of butyrylcholinesterase

The laminar distribution of BChE within the cortex of normal and SDAT cases is shown in figure 14 and analysed in table 12. The normal distribution of BChE is almost the reciprocal of that for AChE. Thus, the lowest activities are found associated with the uppermost layers where the activity is fairly evenly distributed, whilst there is a striking increase in levels in layer VI which continues into the white matter where the highest activities are found. Although the distribution above layer VI is relatively even, there is an apparent rise in activity associated with layer IV/V.

In the SDAT group, the level of activity at each cortical level is apparently higher than the corresponding activity in the normal. Statistically, however, the sum totals (across slices) show that this elevation is not significant ( $F = 0.411$ ; not significant). Qualitatively, the SDAT group did not show the slight elevation in activities observed in the normal cortex in layers IV/V, and the increase in BChE as the white matter is approached was less steep and occurred at a higher level (lower region of layer V as opposed to the mid part of layer VI) than that observed in the normals. However, the overall pattern of distribution was not significantly different between the two groups ( $F = 1.43$ , not significant).

## 3. Distribution of choline acetyltransferase

The laminar distribution of ChAT within the cortex of normal and SDAT cases is shown in figure 15 and analysed in table 12. The normal distribution of ChAT is generally very similar to that observed for AChE with highest activities observed in the upper cortical layers and a trend for decreasing levels as the white matter is approached with, as was the case for AChE, the lowest activities found in the subcortical white

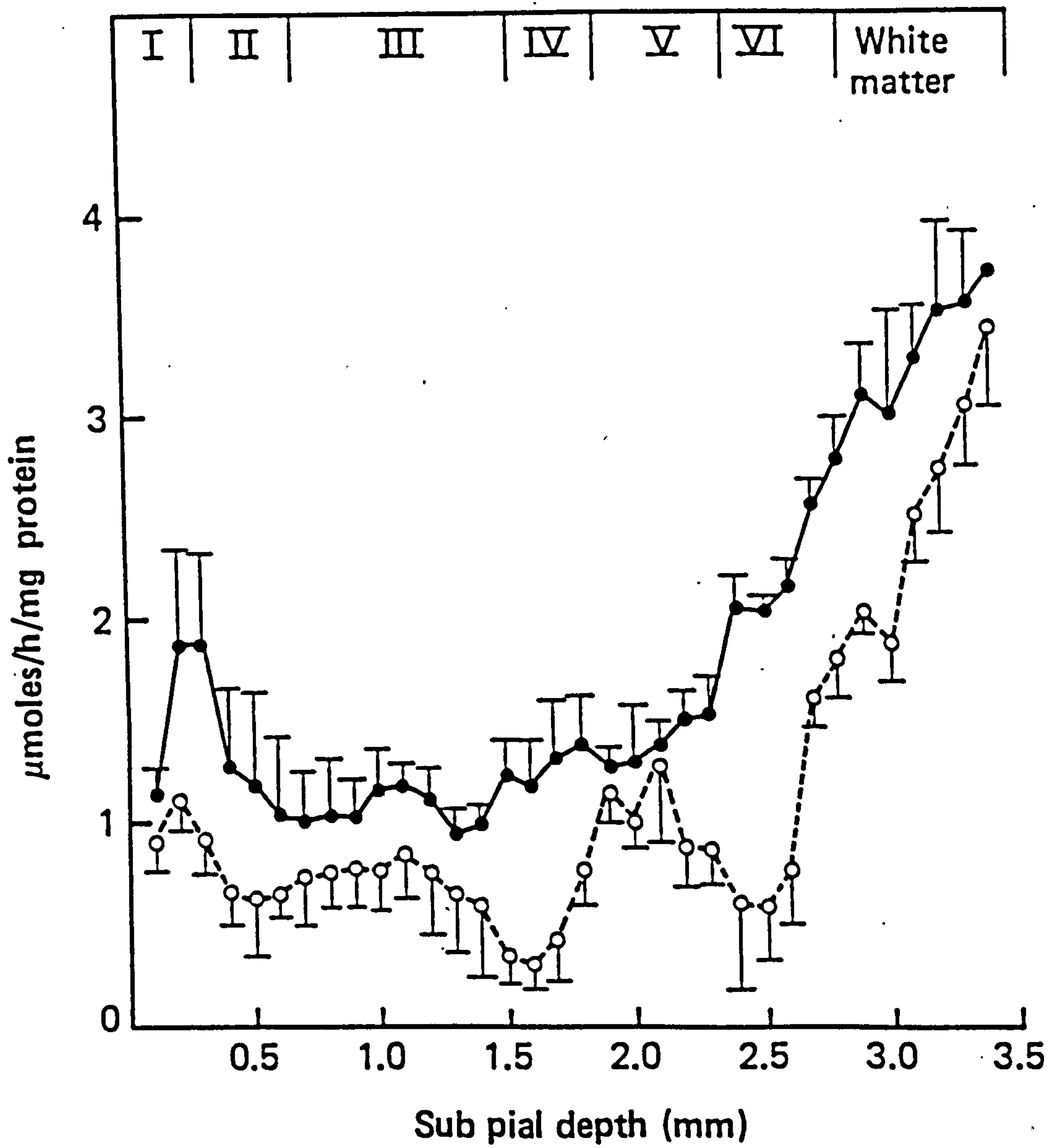


Figure 14. Laminar distribution of butyrylcholinesterase activities in temporal cortex (Brodmann area 21) from normal (O--O) and SDAT (●—●) cases. Points shown are mean values (n=4, both groups) with vertical bars representing the standard errors.



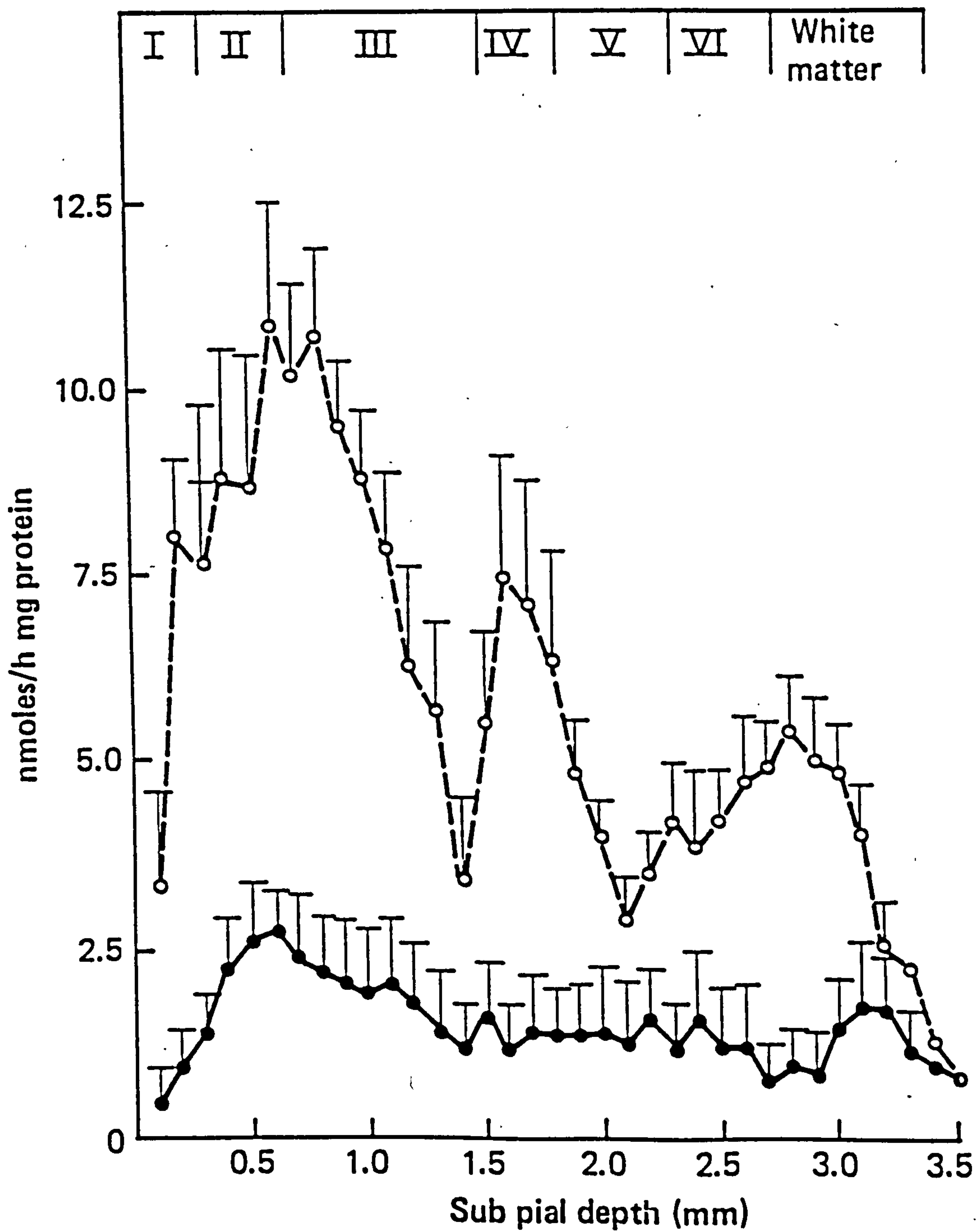


Figure 15. Laminar distribution of choline acetyltransferase activities in temporal cortex (Brodmann area 21) from normal (O---O) and SDAT (●—●) cases. Points shown are mean values (n=4, both groups) with vertical bars representing the standard errors.

matter. More specifically, peaks of activity are associated with the granular layers II and IV, with an additional minor peak found in layer VI, which is again very similar to the normal distribution of AChE. However, in contrast to AChE, which showed a peak of activity in layer III, ChAT activity is relatively low in this region.

In the SDAT cortex there was, as with AChE, substantial reductions in ChAT within the cortex. The sum total (across slices) showed that this reduction was highly significant ( $F=75.02$ ;  $P<0.001$ ), which is in agreement with the reports of reduced ChAT activities observed in cortical homogenates. In addition, the distribution of the remaining activity was greatly altered with loss of the three peaks observed to occur in layers II, IV and VI of the normal. Although a small elevation of residual activity was observed in layer II, the levels observed in this "peak" along with those observed at all other cortical levels, were within one standard error of those found in the white matter where, as with AChE, the activities of both normal and SDAT groups converged.



#### IV. DISCUSSION

The following discussion is divided into two main sub-sections dealing with the laminar distribution in: 1), the normal cortex and 2), the SDAT cortex.

##### 1. Normal laminar distributions

The present microchemical analyses of the mid-temporal cortex in normal elderly cases have clearly demonstrated the value of such a technique, with specific distributions of the different enzymes being observed. Furthermore, the general metabolic enzyme lactic dehydrogenase appears to be concentrated in the pyramidal layers (III and V; Perry et al, 1984a) and is strikingly different from the distributions of AChE, BChE and ChAT, - the individual distributions of which are discussed below - indicating that the localization of these latter enzyme is specific to their functions and not merely a reflection of general metabolic activities.

##### a) Normal distribution of acetylcholinesterase

In the present study, the observation that AChE activity was highest in the upper cortical layers of the temporal cortex is similar to the observations of Pope et al (1952, 1964, 1965) using frontal cortex and the reports that in the rat AChE activity is also relatively enriched in the upper cortical layers of the somatosensory motor (Pope, 1952) and sensorimotor (see Emson and Lindvall, 1979) cortex. In contrast, Okinaka et al (1961) observed that the histochemical AChE-staining was associated particularly with the neurons of layers III and V in various neocortical regions. However, comparisons cannot be readily made between histochemical and biochemical methods since it is very difficult to quantify the histochemical staining of the neuropil and therefore obtain a value of total neuropil plus neuronal cell body activity, which is the parameter being measured biochemically.

When analysed more closely, differences in distribution of activity are found between the present study and the previous studies using frontal cortex (Pope et al, 1952, 1964). Most striking is the absence in the reports of Pope and colleagues of a distinct peak of activity in



layer VI. In addition, they did not observe distinct peaks of activity associated with layers II and IV. The most obvious explanation for this discrepancy is that whilst the present study examined temporal cortex (Brodmann area 21), Pope and co-workers studied frontal cortex (Brodmann area 9), and it is not unreasonable to assume that these different types of cortex may possess subtle chemical differences associated with their different functions. In addition, the sampling technique used by Pope et al (1952, 1964) did not involve as many slices through the cortex as the present study and might therefore be expected to give poorer resolution of any possible peaks of activity.

b) Normal distribution of butyrylcholinesterase

The observation that BChE activity is low in the upper cortical layers and rises as the white matter is approached, with highest levels being found in the subcortical white matter, is in agreement with the previous reports in human cortex using both biochemical (Ord and Thompson, 1952; Foldes et al, 1962) and histochemical (Roessmann and Friede, 1966; Friede, 1967) techniques. Since this distribution is so different from that of AChE - which has highest activities in the upper cortical layers - it would seem unlikely that BChE serves as a precursor of AChE as suggested by Koelle et al (1976a,b, 1977). Such a conclusion is compatible with the observation that in the isolated monkey cortex, AChE activity decreased but BChE remained essentially unaltered (Rosenberg and Echlin, 1965) again indicating that the two enzymes are separately localized.

In the present study an increase of BChE was observed to occur within layer VI and it is interesting to note that Friede (1967) observed that whilst there was little activity in the upper cortical layers, the activity in the lower cortical layers was associated with neurons, and that, in marked contrast to the subcortical white matter, glial cells within the cortex did not stain for BChE. It is therefore probable that at least a part of the activity observed in layer VI is associated with neurons. Similarly, BChE-staining neuronal cell bodies have been identified in layer VI of the macaque monkey cortex (Graybiel and Ragsdale, 1982). The small peaks of activity found in layers I and IV/V may correspond to the myelin found associated with the axonal plexus of layer I and the myelin bands of Baillarger found in layers IV and V. Thus, although Friede (1967) reported that, in contrast to the situation in the white matter, BChE activity was not associated with the glial



cells within the cortex, it is nevertheless possible that the oligodendroglia cells that form the myelin sheath contain a small amount of BChE activity that, although not detected by Friede's histochemical techniques, could be distinguished using the more sensitive biochemical methods employed in the present study.

c) Normal distribution of choline acetyltransferase

The general pattern of distribution of ChAT is similar to that of AChE with the highest levels of activity associated with the upper cortical layers. Similarly, ChAT activity has been reported to highest in the upper cortical layers of human temporal (Amaducci et al, 1981), frontal and cingulate (Lang and Henke, 1983) cortex and in the sensorimotor cortex of the rat (see Emson and Lindvall, 1979).

More specific analysis of the activity of ChAT at different cortical levels shows that distinct peaks of activity are associated with the granular layers II and IV. Similarly, Amaducci et al (1981) observed distinct peaks of cholinergic activity associated with the granular layers of human temporal cortex. Amaducci's study used temporal cortex taken from Brodmann area 22. Since this type of cortex has richer granular layers than the Brodmann area 21 cortex used in the present study (see Brodal, 1969), Amaducci and colleagues observed better defined peaks of ChAT activity than observed here, as would be expected if ChAT was preferentially localized in the granular layers. In contrast to the present report, however, Amaducci et al (1981) observed no peak of activity associated with layer VI and this discrepancy could again be due to the different types of temporal cortex used in the two studies.

d) General distribution of cortical cholinergic activities

Whilst the overall association of the cholinergic-related enzyme activities with the upper cortical layers matches reasonably well with the distribution of cholinceptive cells identified electrophysiologically in the rat and cat cortex (see Emson and Lindvall, 1979), it is interesting to note that the activities of AChE and ChAT are not distributed in parallel through the depth of the human cortex. Similarly, Johnston et al (1981b) have reported that the AChE and ChAT activities were not coincident through the depth of the rat sensorimotor cortex. Thus, although in both the human and rat cortex regions containing relatively high ChAT activities also contain relatively high AChE activities, the converse is not true i.e. areas containing



relatively high levels of AChE do not necessarily contain high levels of ChAT. It is therefore possible that in the results obtained here for human temporal cortex, the high level of AChE activity observed to occur in layer III, but not accompanied by high ChAT activity, is probably not associated with presynaptic cholinergic structures, particularly since a considerable portion of this activity is retained in SDAT.

Since the upper cortical layers are associated with afferent fibres characterised by rich axonal arborizations (Brodal, 1969), the localization of AChE and ChAT in the upper cortical layers is consistent with the primary source of cholinergic activity being an afferent input from sub-cortical regions, the major one of which appears to be the nucleus of Meynert (chapter 1, section II.3.c). In addition, the cortical noradrenaline, dopamine and serotonin neuronal systems which, like the ACh system, are also mainly extrinsic to the cortex (with cell bodies located in the locus coeruleus, the mesencephalic A10 cell group, and raphe nuclei respectively), also appear to be located preferentially in the upper cortical layers of other mammals, which again suggests that these layers may have a predominantly receptive function (Emson and Lindvall, 1979; Reader et al, 1979; Lamour et al, 1983). Interestingly, in an extension of the present study, glutamic acid decarboxylase (GAD) - a reliable marker of the GABA neuronal system, which is thought to be mainly intrinsic within the neocortex (Emson and Lindvall, 1979) - was also found to be associated mainly within the upper cortical layers (Perry et al, 1984a).

The observation in the present study, of a peak of both AChE and ChAT activity in layer VI could correlate with the presence of cholinergic interneurons which have recently been tentatively identified histochemically by the presence of intensely AChE-positive cell bodies (J.M. Candy and R.H. Perry, personal communication). It is possible that this putative intrinsic cholinergic activity found in layer VI is comparable with the AChE-rich neurons of layer VI of the cat cortex (Krnjevic and Silver, 1965) which, it has suggested, may represent a population of intrinsic cholinergic neurons (Jordan and Phillis, 1972). However, the presence of cholinergic cell bodies within the cortex remains an area of controversy. Thus, the observations that cortical slab isolation, which severs the afferent input to the cortex, does not lead to complete depletion of AChE and ChAT (chapter 1, section II.3.c) and a neurophysiological response to ACh remains have been interpreted as indicating the presence of intrinsic cholinergic neurons. On the other



hand, the retention of cholinergic-related enzyme activity and the neurophysiological response to ACh have been hypothesised to be due to a possible peripherally-derived autonomic cholinergic innervation of cortical blood vessels (Fibiger, 1982; see also Estrada et al, 1983). In addition, injections of kainic acid into the cortex of rats have been reported to result both in no reduction or alternatively lead to a 30% loss of cortical ChAT activity (chapter 1, section II.3.c).

Immunohistochemical localization of ChAT-containing cell bodies within the cortex would appear to offer the best method of resolving the controversy yet even here results are conflicting. Thus, although initial reports of immunochemically localized ChAT-containing cell bodies within the cortex (McGeer et al, 1974) were discounted in view of an insufficiently pure ChAT-antigen (see Fibiger, 1982), the recent use of more specific antibodies has lead to reports suggesting that not only are ChAT-containing cell bodies present in the cortex of the rat (Ross et al, 1981; Eckenstein and Thoenen, 1983; Houser et al, 1983) but the majority also contain vasoactive intestinal polypeptide (Eckenstein and Baughman, 1984). In contrast, other groups have reported that intrinsic cholinergic neurons are not present in the cortex of either the rat, cat or monkey (Kimura et al, 1980, 1981; Armstrong et al, 1983; Hedreen et al, 1983; Satoh et al, 1983). These discrepancies may, however, be due to methodological differences since using a similar antibody but different staining technique to that employed by Armstrong et al (1983) and Satoh et al (1983) an intrinsic neuronal population has been recently identified in the rat cortex (see Wainer et al, 1984).

## 2. Laminar distributions in SDAT

The observation that in SDAT the thickness of the cortical grey matter is not significantly different from the normal contradicts the classical concept that in the disease there is a thinning of the cortical grey matter ribbon (Corsellis, 1976; Terry, 1976). However, similar observations have also been made by other groups (Terry et al, 1981; DeKosky and Bass, 1982). In addition, the retention of the six-layered structure of the cortex, along with the near normality of the laminar distribution of LDH activity (Perry et al, 1984a), also suggest that there is not a gross breakdown of cortical structure and function. Indeed, it is suprising that despite the presence of numerous neuro-



pathological changes (up to 50% of the cortex may become involved by plaques; Tomlinson, 1977, 1980) major aspects of cortical structure and neurochemical activity are retained which suggests that, at least in the senile group (in which the neuropathological changes tend to be quantitatively less severe than in the presenile group; chapter 1, section I.2), the disease process is relatively selective.

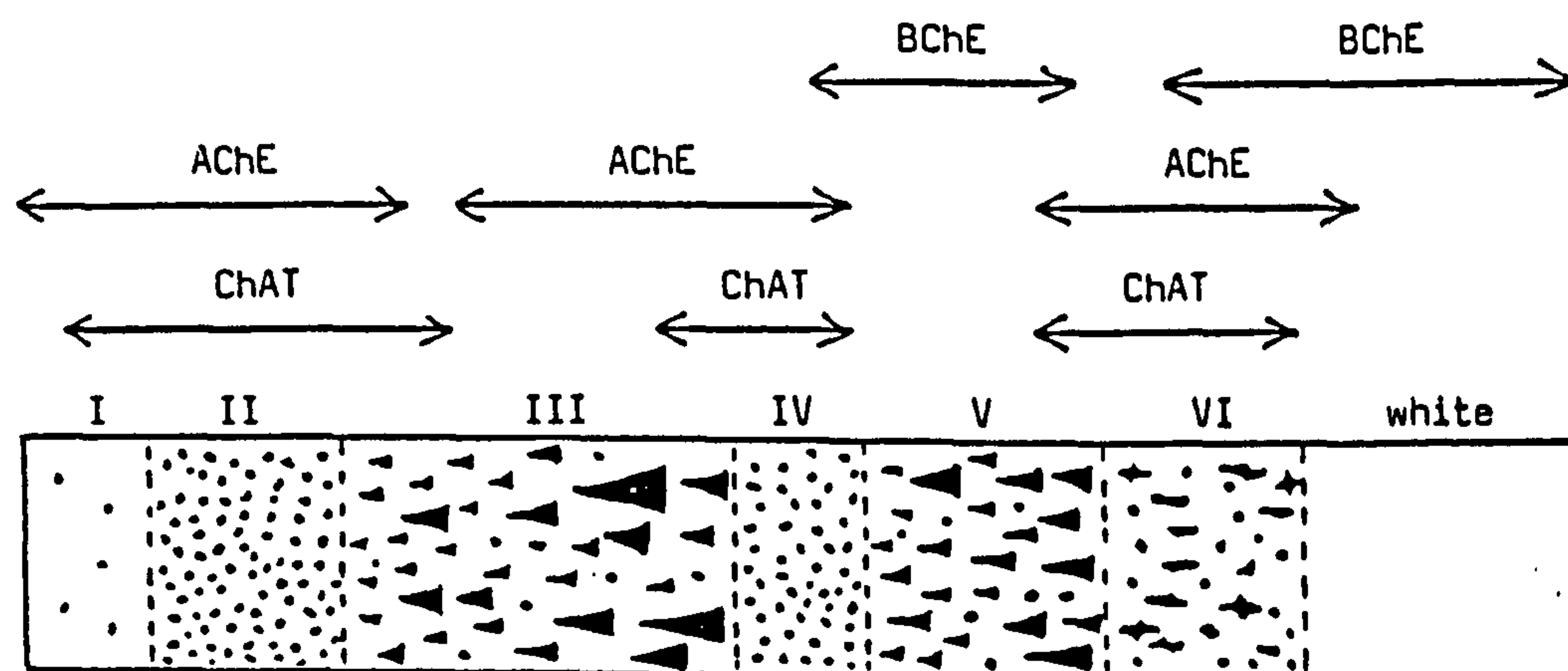
Figure 16 summarises the observations of the present study and compares these with the laminar involvement of other parameters in SDAT. The specific alterations in the distributions of AChE, BChE and ChAT will be discussed below with reference to both normal and pathological features of the cortex.

#### a) Distribution of acetylcholinesterase in SDAT

The present observation of reduced AChE in all cortical layers is in good agreement with the work of Pope et al (1964, 1965) who showed a moderate reduction in AChE in layers II to V (data for layer I was not given). However, in contrast to the reduction observed in the present study, Pope et al (1964) reported an increase in AChE in layer VI of the demented cortex. This discrepancy may be due to the fact that Pope et al (1964, 1965) did not use an inhibitor of BChE in their assay procedure and it may be possible that they observed an increase in BChE, rather than AChE, activity in layer VI which was not observed in the present study. Alternatively, the increased activity in layer VI may indeed be due to AChE activity indicating that the involvement of the cholinergic system is different in the temporal cortex of older subjects (present study) and the frontal cortex of younger cases (Pope et al, 1964, 1965). It is therefore interesting to note that the cholinergic involvement has been reported to be different in the temporal and frontal cortex of younger and older subjects. Thus, Rossor et al (1981a, 1984) have reported that the cholinergic system is affected in the temporal cortex of patients both younger and older than 79 years of age, whilst the cholinergic system in the frontal cortex only appears to be reduced in the patients younger than 79. However, whilst this is a possible explanation, reduced cholinergic activities in the frontal cortex have been reported, in contrast to the work of Rossor et al, in patients with mean ages greater than 80 years old (Bowen et al, 1976; White et al, 1977; Candy et al, 1983).

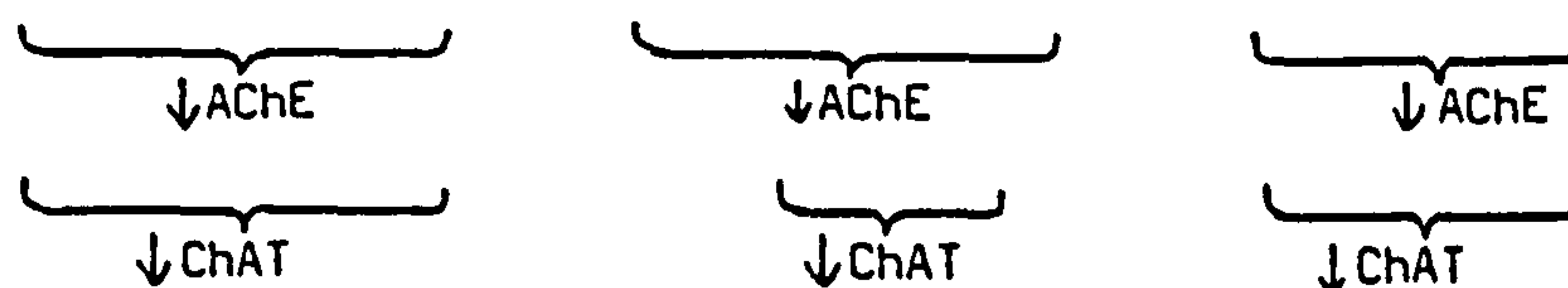


Normal distributions  
in present study

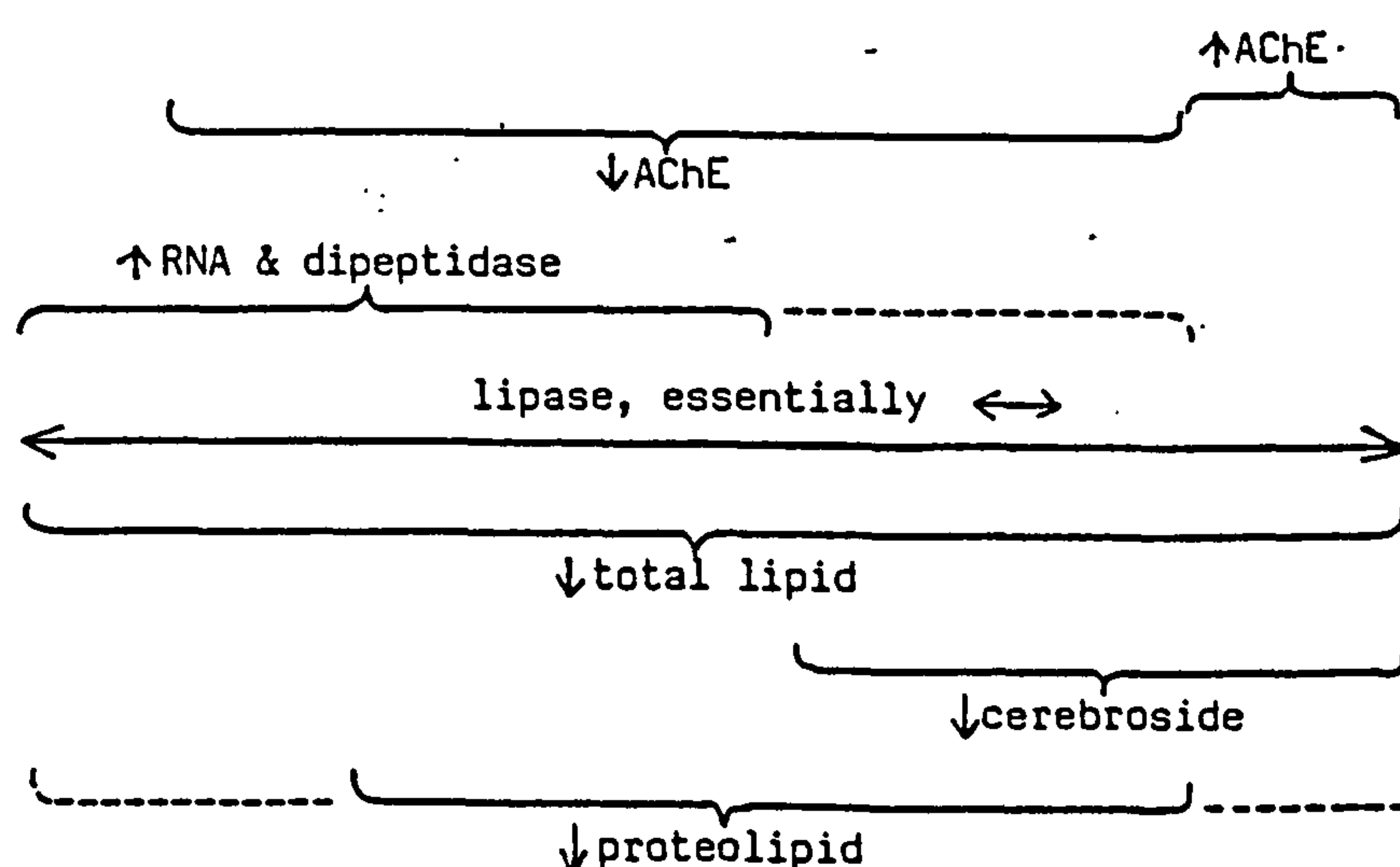


Observations in SDAT

Present study



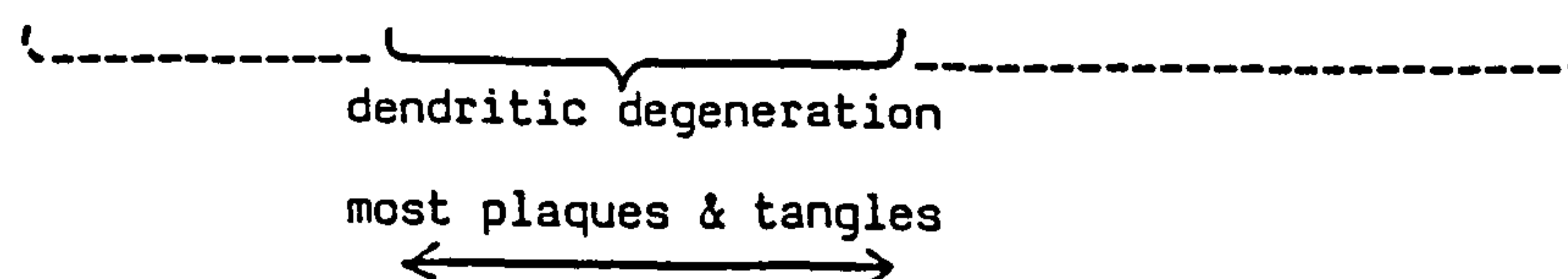
Pope et al, 1964,  
1965



Colon, 1972



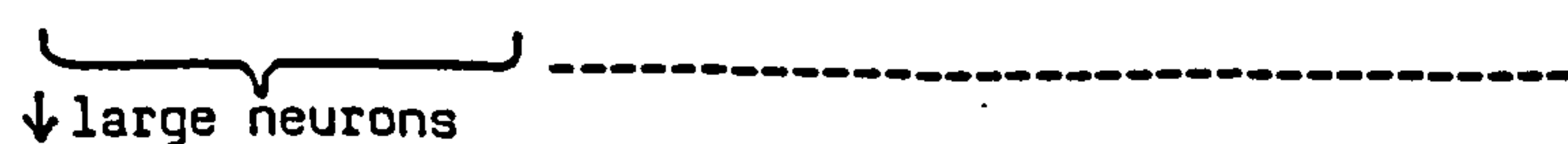
Paula-Barbosa  
et al, 1980



Schechter  
et al, 1981



Terry et al,  
1981



DeKosky and Bass  
1982

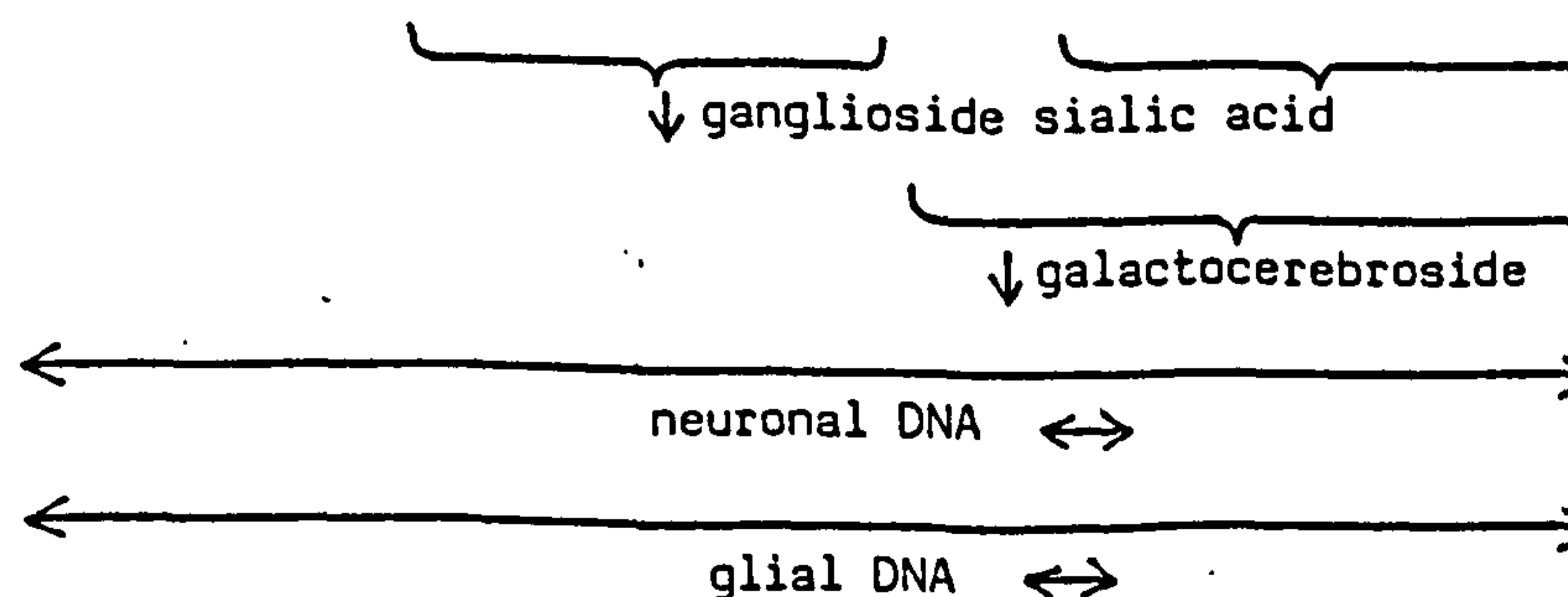


Figure 16. Schematic representation of the laminar distribution of morphological and biochemical abnormalities in the cortex of SDAT subjects. Dashed lines represent regions of less severe changes.

#### b) Distribution of butyrylcholinesterase in SDAT

The observation that total levels (across slices) of BChE are not statistically different in the SDAT group compared to the normal cortex contrasts with the elevation of BChE reported to occur in SDAT and which correlates positively with the degree of dementia (Perry et al, 1978a,b).

However although, Op den Velde and Stam (1976) have also reported an increased BChE activity, their results are not directly comparable with the present results since they only analysed water-soluble enzyme whereas the present technique measures total membrane-bound and soluble activity.

Furthermore, the work of Perry et al (1978b) is also not strictly comparable with the present results since these results were obtained from the pooled values of tissue taken from each of the four lobes of the cortex. Nevertheless, the results obtained by Perry et al (1978a) using temporal cortex, Brodmann area 21, are directly comparable with those presented here and an explanation for the different results obtained is not therefore apparent.

#### c) Distribution of choline acetyltransferase in SDAT

In contrast to the situation in the normal, the intralaminar distribution of ChAT in SDAT bore little resemblance to that of AChE. Thus, in general, there was a reduction of activity to levels comparable with those found in the white matter suggesting that in SDAT, cholinergic axonal terminal activity is reduced to background levels. Similarly, significant loss of ChAT activity was observed in the upper cortical layers of both frontal and cingulate cortex (Henke and Lang, 1983), although a non-significant was observed in the lower cortical layers.

In SDAT, there was no retention of activity in layer III similar to that observed for AChE which, as discussed earlier, is probably due to the fact that this AChE activity is not associated with presynaptic cholinergic structures. Also, although there was a slight elevation of activity associated with layer II, the normal peak of activity found in layer IV was absent altogether indicating that, whereas there might be a total loss of cholinergic innervation to the inner granular layer, a small residual innervation might still be found in the upper granular layer (layer II).

#### d) Laminar distribution of cortical cholinergic activities in SDAT

Of the various parameters examined in the present study, the greatest difference between patterns in the normal and SDAT were those for AChE



and ChAT (Table 12,  $P < 0.001$ ) and this cholinergic abnormality appears to occur, to a greater or less extent, in all cortical layers. This might not appear to be totally compatible with the idea that the loss of cortical cholinergic activity in SDAT is due to loss of afferent input from subcortical regions (presumably projecting mainly to the upper cortical layers, see section I) and which might, therefore, be expected to lead to a preferential loss of activity from the upper cortical layers. However, when Johnston et al, (1981b) placed kainic acid lesions in the basal forebrain region of the rat analagous to the primate nucleus of Meynert, they also observed a loss of AChE and ChAT from all layers of sensorimotor cortex and therefore, by analogy, the results obtained here are compatible with a loss of cortical cholinergic activity in SDAT due, at least in part, to a loss of input from the nucleus of Meynert. This idea is supported by the histochemical observation of a loss of AChE-staining fibres in the cortex in SDAT (Perry et al, 1980, 1983a; Candy et al, 1983). Furthermore, since histochemical AChE-staining is lost from the putative cholinergic cell bodies in layer VI (J.M. Candy and R.H. Perry, personal communication), it would appear that the cholinergic deficit in SDAT is associated not only with a loss of nerve fibres from underlying neurons, but also extends to the intrinsic neuronal population. This intrinsic cholinergic neuronal population is presumably not, however, analogous to the VIP-containing intrinsic neurons identified in the rat cortex (Eckenstein and Baughman, 1984) since levels of this peptide do not appear to be altered in SDAT (chapter 1, section I.7.b.iii).

e) Distribution of cholinergic abnormalities in SDAT with respect to the laminar involvement of other parameters

In the extended study of the laminar distribution of neurochemical activities in SDAT (Perry et al, 1984a), the GABA neurotransmitter system as judged by levels of GABA itself did not appear to be involved at any level of the cortex. However, the relatively high levels of GABA in the white matter suggests that measurements of GABA itself may not be a very specific marker of this neuronal system. Whilst GAD is a better marker, it is however, subject to the influence of agonal status (chapter 1, section I.7.b.i) and was therefore not studied in the SDAT group.

The only neuropeptide to date that has been found to be involved in SDAT is somatostatin, which has been found to be consistently reduced in the neocortex. This has led to the controversial idea (chapter 1,



section I.7.b.iii) that somatostatin and ACh may be localized within the same nerve terminals. However, the results presented here along with those showing the laminar distribution of somatostatin in SDAT (Perry et al, 1983a) suggest that this is unlikely for two reasons; firstly, the normal distribution of somatostatin does not parallel that of the cholinergic-related enzyme activities and secondly, in SDAT, cholinergic activity is lost mainly from the upper cortical layers whereas somatostatin is lost mainly from the lower layers.

Of the other biochemical and morphological changes reported to occur intracortically in SDAT, the majority appear to be associated with the lower cortical layers (see fig 16). For instance biochemical measurements indicate that ganglioside sialic acid, which is enriched in neuronal plasma membranes, is lost from layers III, V and VI (DeKosky and Bass, 1982) and proteolipid is lost from layers III to V (Pope et al, 1964, 1965) - which was noted to coincide with the regional of maximal structural changes (Pope et al, 1964). With respect to the distribution of morphological changes in SDAT, Paula-Barbosa et al (1980) observed that although degenerating dendrites were observed in all cortical layers, degeneration was greatest in layers III and IV, where they also recognised that neurofibrillary tangles and senile plaques were most abundant. When total neuronal cell counts were performed the greatest loss appears in the lower layers (Colon, 1973) - although Terry et al (1981) observed that the greatest loss of large neurons occurred in the first quarter of the cortex lying below the molecular layer - and more specifically Corsellis (1976) states that neuron loss occurs particularly in layers III and V.

It would therefore seem that the majority of structural and neuropathological abnormalities do not occur in parallel with the cholinergic abnormalities. Thus, whilst loss of cholinergic activity does occur in the lower layers, the quantitatively greatest loss of cholinergic activity occurs in layer II, which is structurally relatively intact, suggesting an indirect relationship between the morphological and cholinergic abnormalities in SDAT cortex. This indirect relationship between the cortical morphological and cholinergic abnormalities in SDAT has been further supported by the observation that in patients suffering from Parkinson's disease along with cognitive impairment, reduced levels of both AChE and ChAT were observed in the cortex regardless of whether or not Alzheimer-type neuropathology (senile plaques and neurofibrillary tangles) was present (Perry et al, 1983b).



In conclusion, the neurochemical study of cortical lamination provides a novel approach to assessing the involvement of particular neuronal systems in disorders such as SDAT. Such a technique allows neurochemical observations to be correlated with structural and pathological aspects of the cortex in a way not possible using standard biochemical techniques. Using this approach it is found that the cortical cholinergic-related enzyme activities are associated primarily with the granular layers, which are thought to have a mainly receptive function. This is in agreement with the hypothesis that a major component of cortical cholinergic activity is derived from the sub-cortical nucleus of Meynert. In addition, it has been possible to identify putative cholinergic intrinsic neurons in cortical layer VI, which may also be involved in the pathological processes of SDAT. Furthermore, the results presented here suggest that not only is BChE probably not involved in at least the majority of the cortical cholinergic neurotransmission, but also that, contrary to previous reports, it does not appear to be involved (at least in the temporal cortex) in the pathology of SDAT.

With respect to SDAT, it appears that the cholinergic deficit does not correspond directly with the structural and pathological changes that occur in the cortex. It will therefore be worthwhile applying such techniques to other extrinsic and intrinsic transmitter systems to see if they are more intimately involved in the pathological processes of SDAT.

Chapter 3:

MOLECULAR FORMS OF ACETYL- AND BUTYRYL-CHOLINESTERASE  
IN THE NORMAL AND PATHOLOGICAL CENTRAL NERVOUS SYSTEM



## 1. INTRODUCTION

Studies of the neurochemistry of the cholinergic deficit in senile dementia of the Alzheimer-type (SDAT) have generally been based upon measurements of total activities present in crude homogenates. In the present chapter, a more detailed analysis of the involvement of the cholinesterases was performed by studying the distribution of the different molecular forms of cholinesterases (chapter 1, section II.9) in the normal central nervous system (CNS) and comparing the distribution observed in the normal with that seen in the cortex and nucleus of Meynert of SDAT subjects. In addition, the molecular forms of cholinesterases were investigated in the cortex of Parkinson's disease patients with dementia and are discussed in relation to the results observed in the SDAT brain. Finally, as a model of central cholinergic deafferentation analogous to that suggested to occur in SDAT, the molecular forms of AChE were measured in the hippocampus of rats with lesions of the septo-hippocampal pathway.

Before these analyses were performed, it was necessary to establish the suitability of human postmortem brain tissues for such studies. Thus, whilst the total levels of AChE and BChE are relatively stable postmortem (chapter 1, section II.4.a) it is nevertheless possible that the distributions of the different molecular forms may alter as a result of proteolytic activity within either the intact tissue or, alternatively, as a result of the exposure of the cholinesterases to proteolytic enzymes during the homogenising and solubilization procedures. Therefore a series of preliminary experiments were conducted to establish that the molecular forms of both AChE and BChE were stable both in the intact tissue and during the solubilization procedures used.

The results presented here demonstrate that in the neocortex of SDAT subjects there is a selective loss of one particular form of AChE, but no alteration in the forms of BChE. The analyses of the AChE molecular forms in the subcortical nucleus of Meynert in SDAT showed that, unlike the cortex, all the forms are reduced and by similar amounts. In addition, to determine whether or not the reported cholinergic involvement in Parkinson's disease (Ruberg et al, 1982; Dubois et al, 1983; Perry et al, 1983b) is similar to that found in SDAT, analyses of the molecular forms of AChE and BChE were carried out in the neocortex of Parkinson's disease patients who, despite the absence of Alzheimer-type

neuropathology, were also demented. The changes observed in this situation were identical to those observed in the SDAT subjects and suggest that the changes in the cortical cholinergic system in these two disorders are similar.

Finally, since the changes that occur in the cholinergic system of the rat hippocampus after lesions of the septo-hippocampal pathway - for example reduced levels of AChE (Srebro et al, 1973), choline acetyltransferase (Lewis et al, 1967), acetylcholine and high-affinity choline uptake (Kuhar et al, 1973) but unaltered levels of postsynaptic receptor binding (Yamamura and Snyder, 1974) - are similar to the changes that occur in SDAT, the molecular forms of AChE were also examined in the hippocampus of lesioned rats. The changes observed after the central cholinergic deafferentation produced by such lesions were similar, but not identical, to the changes observed in the SDAT cortex and are compatible with the suggestion that the loss of cortical cholinergic activity in SDAT is due to a degeneration of the afferent cortical cholinergic input.

The results and discussion are divided into three main sub-sections that describe: 1, preliminary experiments carried out to establish the conditions of centrifugation and the suitability of human postmortem tissue for the study of cholinesterase molecular forms; 2, the distribution of molecular forms of cholinesterases in the normal CNS and 3, the distribution of molecular forms of cholinesterases in the CNS in different pathological situations. The results are discussed with respect to both pathological and more fundamental aspects of central cholinergic neurotransmission and the relationship of BChE not only to AChE but also to the central cholinergic system. Some of the results described here have been presented elsewhere (Atack et al, 1983b,c).



## II. MATERIALS AND METHODS

The present section is divided into three sections that describe: 1, the subjects and procedures used for the sampling of tissue used in the analysis of molecular forms in the normal and pathological CNS; 2, the preliminary experiments carried out to establish the suitability of human postmortem tissues to the study of cholinesterase molecular forms and 3, the density gradient centrifugation techniques used.

The chemicals used in the present study were mainly obtained from the Sigma Chemical Company (Poole, England) and included: aldolase (rabbit type IV), alkaline phosphatase (bovine; type VII), aprotinin (bovine lung), bacitracin, benzamidine hydrochloride, catalase (bovine liver), cytochrome c (horse heart; type VI), ethopropazine hydrochloride, ethylenediaminetetraacetic acid (EDTA; Sigma grade), N-ethylmaleimide,  $\beta$ -galactosidase (E. coli; grade VIII), haemoglobin (bovine; type I), leupeptin (synthetic), nicotinamide adenine dinucleotide (NAD, yeast; grade III), pepstatin, sucrose (grade I), trypsin inhibitor (lima bean; type II-L), trypsin inhibitor (chicken; type IV-0), trypsin inhibitor (soya bean; type I-S) and thyroglobulin (type I). Alcohol dehydrogenase (horse liver) and O-nitrophenyl- $\beta$ -D-galactopyranoside were obtained from Koch-Light (Haverhill, England) and ethanol and hydrogen peroxide (AnalaR and Aristar grades respectively) were purchased from BDH (Poole, England).

The sources of other chemicals used in the present section are described in the preceding chapter.

### 1. Subjects and sampling procedures used in the study of the normal and pathological central nervous system

#### a) Subjects

The normal subjects all died suddenly and were selected on the basis of the lack of a clinical history of psychiatric illness and absence of significant neuropathological changes. The details of the normal subjects used in the analysis of molecular forms in different regions of the CNS are presented in table 13 and the mean plaque counts of the normal subjects used for comparisons with the SDAT and Parkinson's disease patients are presented in tables 14 and 15.

Table 13  
Analysis of the subjects used in the study of the molecular forms of acetyl-  
and butyryl-cholinesterase in different regions of the human central nervous system <sup>a</sup>

	Sex male:female	Age years	Postmortem delay, hours
Caudate nucleus	2/1	76.3 ± 1.2	27.3 ± 5.5
Parietal cortex	2/3	80.0 ± 7.0	26.6 ± 8.8
Nucleus of Meynert	3/2	82.0 ± 5.2	28.6 ± 7.8
Temporal cortex	2/1	81.7 ± 9.5	28.0 ± 10.5
Hippocampus	2/1	79.0 ± 2.7	29.0 ± 3.5
Spinal cord	2/0	62, 70	20, 32
Substantia nigra	1/1	63, 63	14, 24
Cerebellum	2/1	78.7 ± 2.9	28.7 ± 3.7
Fornix	0/2	75, 87	33, 52
Cerebrospinal fluid	3/0	52.3 ± 7.1	<sup>b</sup> N/A

<sup>a</sup> Values shown are mean ± standard deviation when n = 3 or more. When n is less than 3, individual values are shown.

<sup>b</sup> N/A = not applicable



Table 14  
Details of subjects used in the analysis of molecular forms of  
acetylcholinesterase and butyrylcholinesterase in parietal cortex <sup>a</sup>

	Normal	SDAT	Parkinson's disease
Age, years	80.0 $\pm$ 7.0	76.8 $\pm$ 4.2	67.3 $\pm$ 4.9
Sex, male/female	2/3	2/3	3/0
Postmortem delay, hours	26.6 $\pm$ 8.8	27.0 $\pm$ 5.6	21.7 $\pm$ 14.5
Mean plaque count <sup>b</sup>	4.1 $\pm$ 2.9	50.1 $\pm$ 8.4	5.3 $\pm$ 7.2

<sup>a</sup> Values shown are mean  $\pm$  standard deviation

<sup>b</sup> Mean plaque count is the mean of the number of senile plaques in five 1.3mm diameter microscopic fields in each of six different neocortical brain areas (Blessed et al, 1968).

Table 15  
Details of subjects used in the analysis of molecular  
forms of acetylcholinesterase in the nucleus of Meynert <sup>a</sup>

	Normal	SDAT
Age, years	82.0 $\pm$ 5.2	83.7 $\pm$ 4.5
Sex, male/female	3/2	2/1
Postmortem delay, hours	28.6 $\pm$ 7.8	29.0 $\pm$ 8.5
Mean plaque count <sup>b</sup>	3.9 $\pm$ 3.2	45.4 $\pm$ 13.7

<sup>a</sup> Values shown are mean  $\pm$  standard deviation

<sup>b</sup> Mean plaque count is the mean of the number of senile plaques in five 1.3mm diameter microscopic fields in each of six different neocortical brain areas (Blessed et al, 1968).

The SDAT subjects were selected according to the clinical and neuropathological criteria outlined previously (chapter 2, sections II.1 and 3). The Parkinson's disease subjects demonstrated the classical clinical features of the disease (tremor and akinesia) in addition to which a co-existent dementia was also present. Subsequently, these patients were all found to possess Parkinson-type neuropathology (following section). The details of the normal, SDAT and Parkinson's disease cases used in the analysis of molecular forms of AChE and BChE in the parietal cortex are shown in table 14. The normal and SDAT cases are well matched for sex, age and postmortem delay although the Parkinson's disease cases were all male and were younger than both the normal and SDAT subjects. The cases used for analysis of the molecular forms of AChE in the normal and SDAT nucleus of Meynert are presented in table 15 and are comparable in respect to patient sex, age and postmortem delay.

#### b) Neuropathology

The neuropathological procedures used for the identification of Alzheimer-type changes are as described previously (chapter 2, section II.3). Hence, the normal and SDAT groups were characterised by their low and high mean plaque counts respectively (tables 14 and 15) and the presence of large numbers of neocortical neurofibrillary tangles in the SDAT but not normal cases.

The Parkinson's disease patients were confirmed neuropathologically on the basis of the presence of Lewy bodies and absence of pigmented neurons in the substantia nigra. Furthermore, the Parkinson's cases had low mean plaque counts (table 14) and few neocortical neurofibrillary tangles indicating that Alzheimer-type pathology was not present.

#### c) Sampling of human material

The procedures used for the sampling of the human postmortem brain are similar to those described earlier (chapter 2, section II.2). Briefly, the left hemisphere was sliced coronally for biochemical sampling whilst the whole right hemisphere was fixed in neutral formalin for subsequent neuropathological examination. The samples removed from the various regions of the CNS - except the nucleus of Meynert, see below - were all dissected fresh and frozen and stored in screw-top polypropylene tubes in liquid nitrogen. These included the parietal cortex, (Brodmann area 40) which was removed from coronal level 24/25 (R.H. Perry and A.E. Oakley, in preparation), the rostro-medial portion



of the head of the caudate nucleus (coronal level 9/10), mid-temporal cortex (Brodmann area 21, coronal level 16/17), the hippocampus (dentate gyrus to subiculum) dissected free from the hippocampal gyrus (coronal level 18/19), spinal cord (either mid cervical or thoracic region), the mid portion of the substantia nigra removed from the mid zone of the mesencephalon, the inferolateral portion of the cerebellar hemisphere and the ascending and horizontal portion of the fornix dissected free of choroid plexus (coronal level 16/18).

In order to obtain discrete samples of the nucleus of Meynert, a micropunch technique was used (a detailed description of which is available elsewhere; Perry et al, 1984b). In brief, blocks of the basal forebrain that included the nucleus of Meynert and the adjacent structures shown in figure 17 were removed from coronal sections of fresh brain, sealed in polythene bags, rapidly frozen in melting arcton and then stored at either  $-70$  or  $-120^{\circ}\text{C}$ . The frozen, unfixed tissue was subsequently serially sectioned into thick (150-200 $\mu\text{m}$ ) or thin (40-50 $\mu\text{m}$ ) sections. The thin sections were used for the histochemical demonstration of AChE using the method of Karnovsky and Roots (1964), and a typical distribution of activity is shown in figure 5. Three days or less before extraction of the molecular forms, two samples were removed from an adjacent frozen thick section, using a stainless steel micropunch of 1-2mm internal diameter, corresponding to the AChE-rich nucleus of Meynert and stored in capped polypropylene tubes at  $-70^{\circ}\text{C}$ .

In addition to the postmortem material, three samples of cerebrospinal fluid (CSF) were obtained by lumbar puncture from three subjects who had no signs of psychiatric illness, but were suffering from benign cranial hypertension (two patients) and a lumbar disc ailment (one patient). The CSF was stored in liquid nitrogen until analysed.

#### d) Experimental procedures in rat lesioning studies

To investigate the changes in the molecular forms of AChE that occur as a consequence of central cholinergic deafferentation, a series of experiments were carried out on rats in which the cholinergic input to the hippocampus from the septum had been destroyed. Thus, thermocoagulative lesions were placed in the fornix and after 14 days, in which time cholinergic elements distal to the lesion degenerate, the molecular forms of the remaining AChE activity were analysed.

All the rats were 180-200g male Wistar albino rats aged 6 months. Prior to lesioning of the fornix, the animals were anaesthetised using a

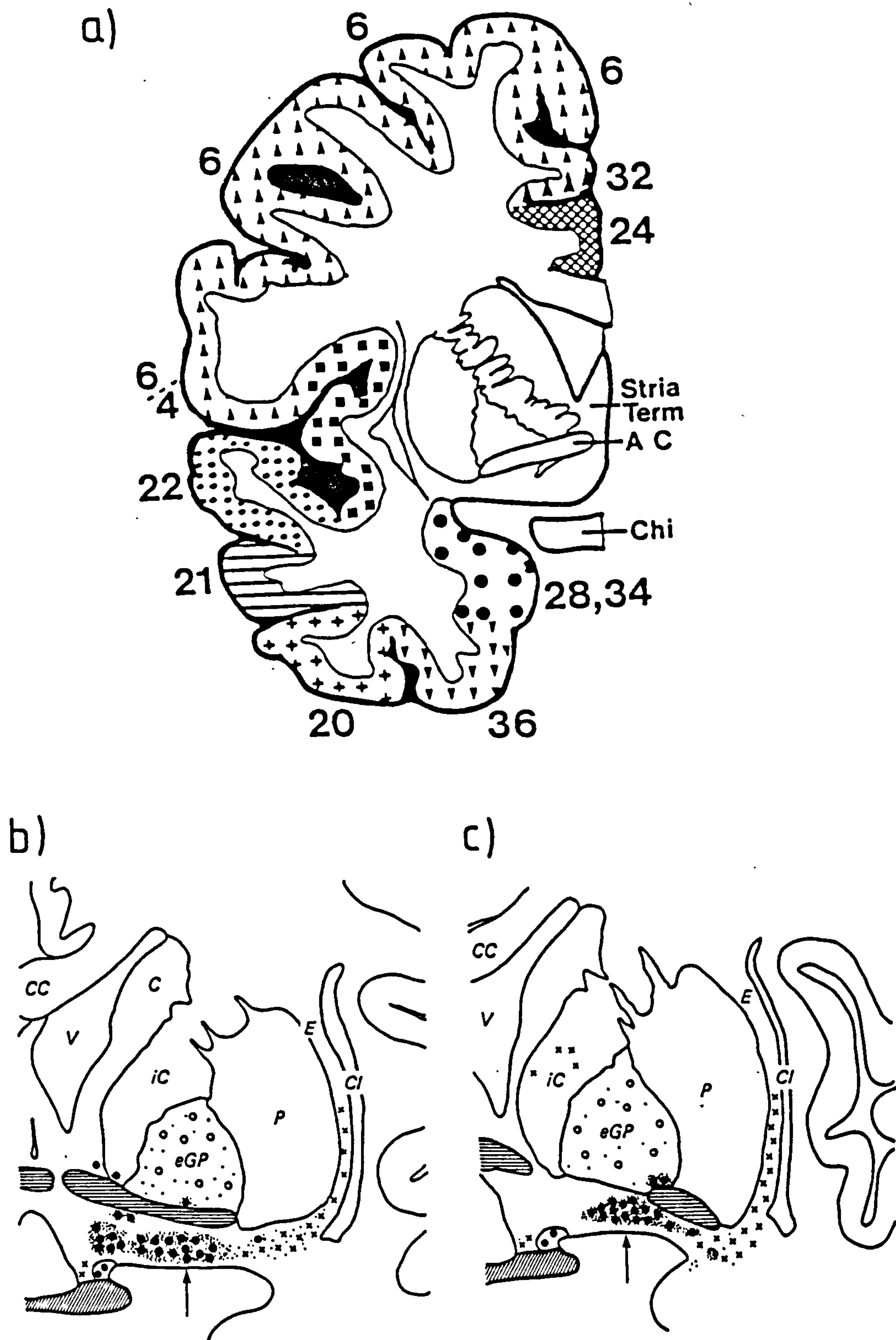


Figure 17. Diagram illustrating the location of tissue sampled from the nucleus of Meynert.

a) Diagram of the whole coronal slice at the level from which material was taken. Abbreviations; Stria Term, stria terminalis; A C, anterior commissure; Chi, optic chiasm; numbers indicate Brodmann areas.

Precise rostral b) and caudal c) levels between which tissue was sampled. Filled and open circles represent intensely and moderately stained cell bodies respectively; dots show AChE-stained neuropil and crosses AChE-stained fibres C, caudate nucleus; CC, corpus callosum; Cl, claustrum; E, external capsule; eGP, external globus pallidus; iC, internal capsule; P, putamen; V, ventricle. In addition, horizontally shaded areas represent the anterior commissure and the diagonally shaded areas show the position of the optic tract.



1:10 dilution of Nembutal (sodium pentobarbitone) at a dose of 1ml per 200g body weight administered i.p. The anaesthetised rats were then placed in a Kopf stereotaxic instrument and the scalp incised and the skull cleared of connective tissue. A hole, with co-ordinates +6.0mm and 0.4mm in the anterior-posterior (relative to the interaural line) and lateral axes respectively, was then placed in the skull using a dental drill and a Kopf electrode, with thermistor probe tip and feedback temperature control, was lowered through the hole to a position +2.2mm in the vertical axis. The electrode was left in the brain for five minutes (to allow the brain to regain its original undistorted shape) after which a current was passed through the electrode (using a Kopf lesion generator, model RFG4) for 1 minute, sufficient to provide temperature of 80°C at the tip of the electrode. The electrode was then withdrawn and the hole in the skull was sealed with bone wax. The scalp was sewn together and the antibiotic powder Acramide was sprinkled over the wound.

The rats were then housed individually and fed ad lib for 2 weeks before being sacrificed by cervical crush. The brain was then removed and the mid-hippocampus dissected and stored at -70°C for subsequent biochemical analysis. Confirmation of the extent of the lesion was performed on the remainder of the brain which was fixed in formal calcium and examined histologically using the Nissl stain. The position of a typical lesion is shown diagrammatically in figure 18 and extended from the septum to the anterior hippocampus. Only operated animals that had been confirmed histologically as having extensive lesions of the fornix were used for subsequent biochemical analyses.

Identical procedures were carried out on an additional series of rats with the only difference being that in these animals no current was passed through the electrode. These animals therefore served as sham-operated controls.

## 2. Preliminary experiments

A series of experiments were carried out to determine not only the sedimentation profile of the sucrose density gradient but also the influence of postmortem delay and storage of extracts on the distribution of AChE and BChE molecular forms. These experiments are described below.

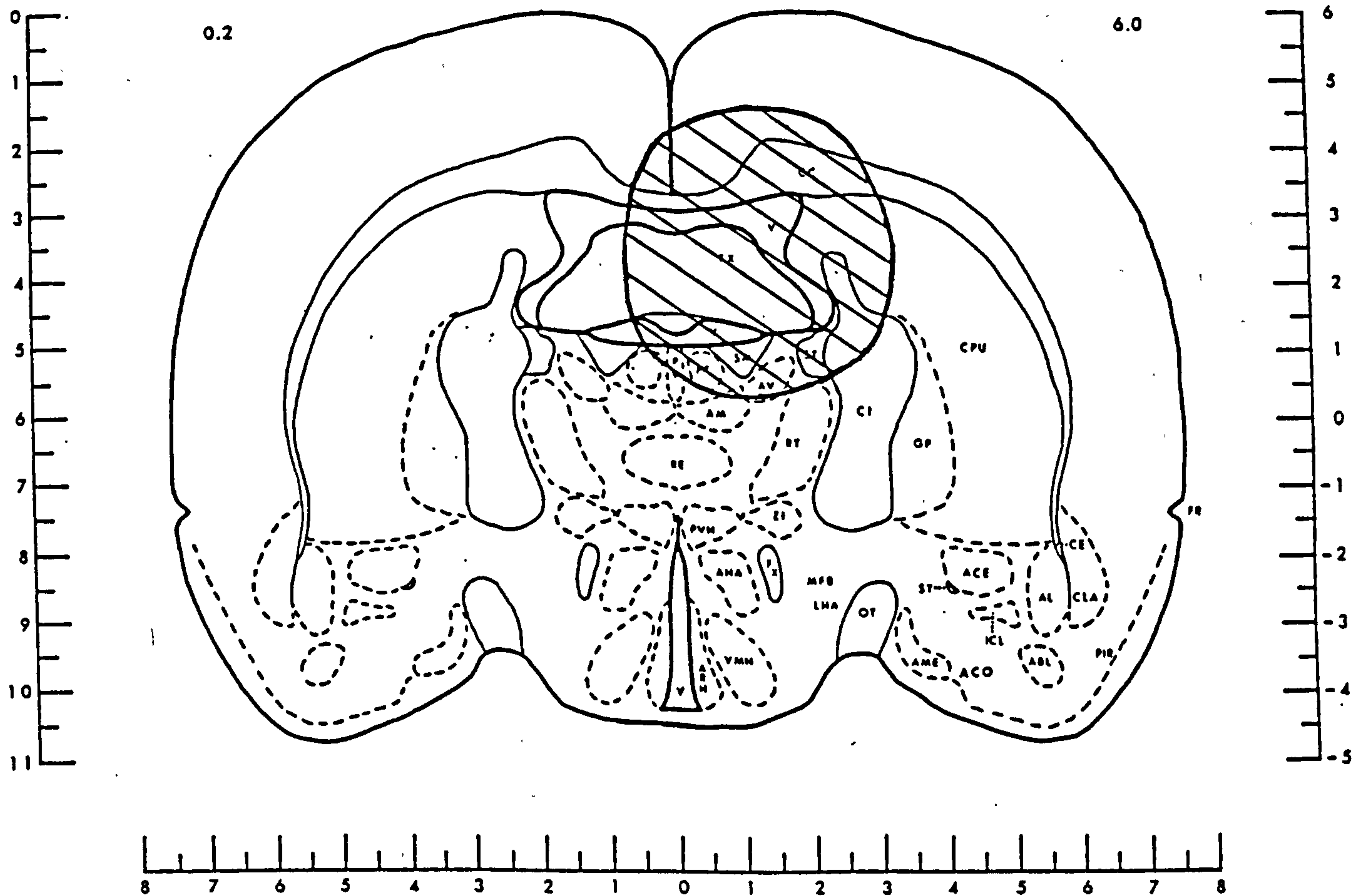


Figure 18. Diagrammatic illustration of the position of the unilateral thermocoagulative lesion. Coronal section of the brain at a level +6.0mm in the anterior-posterior axis relative to the interaural line (Pellegrino et al, 1979) showing the site (diagonal shading) of a typical lesion. The lesion extended from the septum to the anterior part of the hippocampus.



a) Sedimentation profiles of the sucrose density gradients

In order to determine the sedimentation velocity profiles of the two types of gradient used in the present study, one gradient of each type (section 3.c) was loaded with a series of different marker proteins of known sedimentation velocities. Thus, in addition to alcohol dehydrogenase (sedimentation velocity, 4.8 Svedberg units), B-galactosidase (16.0S) and catalase (11.4S) - which were present in the amounts described in section 3.c.iii - 1mg of each of the following proteins were also loaded: cytochrome c (1.7S), aldolase (8.3S), thyroglobulin (19.4S), ovalbumin (3.6S), alkaline phosphatase (6.1S), and haemoglobin (4.2S). The positions on the gradients of alcohol dehydrogenase,  $\beta$ -galactosidase and catalase were measured as described in section 3.d whilst the positions of the remaining proteins was determined by assaying each gradient fraction for protein using the method of Lowry et al (1951), using different concentrations of bovine serum albumin to construct a calibration curve.

b) Postmortem stability of molecular forms of acetyl- and butyryl-cholinesterase

To determine the effects of postmortem delay on the molecular forms of AChE and BChE in the brain, six mice were sacrificed and the brains of three removed immediately and 10% w/v homogenates were prepared, and analysed as described below (section 3). The other three mice were maintained for 7 hours at room temperature followed by 24 hours at 4°C to simulate approximately the postmortem conditions of the human cadaver. After this period the brains of these three mice were removed, extracted and analysed using the same conditions as for the brains analysed immediately after death.

c) Extractions in the presence and absence of protease inhibitors

Since endogenous proteases have been reported to modify the molecular forms of AChE in chick skeletal muscle (Silman et al, 1978) extracts of human temporal cortex were prepared in the absence and presence of various protease inhibitors. For this purpose temporal cortex grey matter was dissected from white matter and finely chopped with a scalpel blade to ensure a homogenous distribution of tissue. Aliquots of the chopped tissue were taken and 10% w/v homogenates prepared and extracted (section 3) in the following media;

- 1) 1M NaCl/10mM phosphate buffer/0.5% v/v Triton X-100, final pH 7.1.

- 2) medium 1) plus 2mM EDTA to inhibit proteases activated by metal ions.
- 3) medium 1) plus 1mg/ml bacitracin; 2mM benzamidine hydrochloride; 5mM N-ethylmaleimide and 20µg/ml pepstatin (Edwards and Brimijoin, 1983)
- 4) medium 1) plus 0.2mg/ml aprotinin; 1.0mg/ml bacitracin; 2mM benzamidine hydrochloride; 5mM EGTA; 5mM N-ethylmaleimide; 40µg/ml leupeptin; 20µg/ml pepstatin; 20µg/ml trypsin inhibitor (chick); 0.1mg/ml trypsin inhibitor (lima bean) and 0.1mg/ml trypsin inhibitor (soya bean), modified from Lai et al, (1983).

d) Effects of storage of frozen extracts upon the distribution of AChE and BChE molecular forms

Since it would have been convenient to perform all extracts at the same time and store the extracts at  $-20^{\circ}\text{C}$  prior to analysis, a series of experiments were carried out to study the effects of storage upon the distribution of molecular forms of AChE and BChE. For this purpose 10% homogenates of both caudate nucleus and temporal cortex were prepared as described in section 3.a and whilst one aliquot of extract was analysed immediately and assayed for both AChE and BChE (temporal cortex) or AChE alone (caudate nucleus), a second was frozen and stored at  $-20^{\circ}\text{C}$  for a period of 4 weeks before being thawed and analysed under identical conditions to those used for the analysis of the "fresh" extracts.

### 3. Analysis of molecular forms of cholinesterases

#### a) Extraction of cholinesterases

The extraction of the molecular forms of cholinesterases from different regions of the CNS were similar in most respects and are described below for the parietal cortex. The main exception to these procedures was the nucleus of Meynert, for which fixed w/v% homogenates were not prepared (although all the other procedures used were as described for the parietal cortex).

For analysis of the molecular forms present in the cortex, the frozen material was thawed slowly at  $4^{\circ}\text{C}$ , the surface membranes removed and approximately 100-200mg of grey matter were carefully dissected from the white and weighed. A 10% w/v grey matter homogenate was then prepared using a homogenising medium consisting of 1M NaCl/10mM phosphate buffer/0.5% v/v Triton X-100, final pH 7.1. The tissue was homogenised, on ice, using 100 passes of a Potter teflon-on-glass homogeniser set at a



speed of 1500rpm. The resulting homogenate was clarified by centrifugation at 4°C for 20 minutes in a MSE High-Speed 18 centrifuge at a speed (13,000rpm) equivalent to 20,000g and the resulting supernatant, representing solubilized AChE and BChE, was used immediately for the analysis of molecular forms. Aliquots of both the supernatant and the original homogenate were retained for subsequent assay to determine the percentage extraction of each enzyme from the homogenate into the soluble form.

For the analysis of molecular forms, all other human CNS regions - except for the nucleus of Meynert; see below - in addition to whole mouse brain and rat hippocampal samples were extracted in a similar fashion. The only differences were the preparation of 5% rather than 10% homogenates of rat hippocampus and the additional presence in the homogenising buffer of 2mM EDTA in order to solubilize the class of asymmetric molecular forms that are not solubilized by high salt concentrations (Gomez-Barriocanal et al, 1981; Barat et al, 1984).

The nucleus of Meynert samples differed from the other samples analysed in that, due to the small amounts (approximately 300µg) of tissue available, it was difficult to prepare a fixed percentage w/v homogenate. It was therefore necessary to measure the protein content of the individual samples in order to analyse extracts derived from comparable amounts of tissue. Therefore to each two-punch sample, 100µl of 1M NaCl/10mM phosphate buffer, pH 7.1 were added and the tissue dispersed by passing the suspension 30 times through the needle of a fixed-needle 100µl syringe (Terumo, Tokyo, Japan). From the resulting suspension of dispersed tissue three 5µl aliquots were removed for protein determination by the method of Lowry et al (1951) in which varying concentrations of bovine serum albumin, made up in 1M NaCl/10mM phosphate buffer, pH 7.1, were used for the construction of a calibration curve. To the remaining 85µl were added 300µl of 1M NaCl/10mM phosphate buffer/0.64% v/v Triton X-100/2.56mM EDTA, final pH 7.1 (giving a final concentration in the homogenising medium of 1M NaCl/10mM phosphate buffer/0.5% Triton X-100/2mM EDTA) and this was then homogenised and clarified by centrifugation at 20,000g (see above) and the supernatant was analysed for the molecular forms of AChE but not BChE which, due to the small amounts of tissue analysed, was below the limits of detection of the assay procedure used. Aliquots of the original homogenate and the supernatant were retained to determine the percentage extraction of AChE from the homogenate into the soluble form.



b) Solubility characteristics of parietal cortex molecular forms

In order to determine the proportions of the molecular forms of AChE and BChE of normal parietal cortex that were asymmetric or globular and soluble or membrane bound, tissue was extracted in the absence and presence of high salt concentrations and detergent respectively.

i) Extraction in the absence and presence of high salt concentrations

Although the majority of the cortical (and other regions of the CNS) AChE was found to have a sedimentation velocity of approximately 10S and, in comparison with other species (Massoulie and Bon, 1982), probably represents the tetrameric globular ( $G_4$ ) form, it is nevertheless possible that this form may be the tetrameric asymmetric ( $A_4$ ) form. Thus, in the human muscle (Carson et al, 1979), as well as in other species (Massoulie and Bon, 1982), the  $G_4$  and  $A_4$  molecular forms have similar sedimentation velocities. However, since asymmetric forms can be distinguished from globular forms by their requirement for high salt concentrations for their extraction (Bon et al, 1979; Grassi et al, 1982), extraction of tissue in low salt concentrations solubilises only globular forms whilst re-extraction of the tissue in high salt concentrations solubilises the asymmetric forms. Therefore, a 10% w/v homogenate of normal parietal cortex was prepared, as described in section a) in a low salt (10mM NaCl) buffer (10mM phosphate buffer /0.5% v/v Triton X-100 pH 7.1). This was clarified by centrifugation at 20,000g for 20 minutes and the supernatant, which represented low-salt soluble (globular) AChE and BChE, was removed. The pellet was then re-extracted by homogenisation under identical conditions and in a similar buffer this time containing a high salt (1M NaCl) concentration. The 20,000g supernatant of the re-extracted pellet represented high-salt soluble (asymmetric) AChE and BChE. The extracts of globular and asymmetric molecular forms were then loaded onto separate gradients and analysed as described below (sections c and d).

ii) Extractions in the absence and presence of detergent

In contrast to extractions carried out in the absence and presence of high salt concentrations, in which a distinction can be made between globular and asymmetric forms, it is possible to determine the proportions of any particular molecular form that are soluble and membrane-bound by extracting the tissue in the absence and presence of detergent respectively (Lazar and Vigny, 1980; Grassi et al, 1982).



Therefore, to determine the proportions of AChE and BChE molecular forms that were soluble and membrane bound, extracts of normal parietal cortex were prepared by extraction and re-extraction (see above) in 1M NaCl/10mM phosphate buffer, pH 7.1 in the absence and presence of 0.5% v/v Triton X-100 detergent respectively. The initial extraction in the absence of detergent represented soluble enzyme whilst membrane-bound enzyme is only solubilized by re-extraction in the presence of detergent (Lazar and Vigny, 1980; Grassi et al, 1982). Separate analyses of the soluble and membrane bound enzymes were performed as described below (sections c and d).

### c) Sucrose density gradient centrifugation

#### i) Preparation of sucrose density gradients

Sucrose density gradients were all formed in 5.0ml polyallomer Beckman centrifuge tubes (1.3cm by 5.1cm) and were of two types; in the first type, gradients were made by overlaying equal volumes (1.025ml) of 20, 15, 10 and 5% w/v sucrose solutions made up homogenising medium to a final volume of 4.1ml and leaving the layers to diffuse, for approximately 3 hours, at room temperature until the interface between the layers - which was initially very distinct - was no longer visible (figure 19). After this period the gradients were placed on ice for at least 15 minutes and 0.5ml of 50% w/v sucrose, also made up in homogenising medium, was slowly underlayered at the bottom of the gradient using a long-needled hypodermic syringe. This layer formed a "cushion" of sucrose to prevent any possible pathologically heavier molecular forms pelleting at the bottom of the gradient. The second type of gradient used was a 10-40% w/w sucrose gradient and differed in that not only was the gradient steeper (which results in sharper, better resolved peaks of activity: Griffiths, 1979) but also no sucrose cushion was used. The 10-40% gradients were made by overlaying equal volumes (the exact volume of which varied with the amount of each particular type of extract loaded) of 10, 20, 30 and 40% w/w sucrose solutions, again made up in homogenising medium, and leaving the gradients to form as described for the 5-20% gradients.

#### ii) Centrifugation and fractionation

After the gradients had formed, the appropriate volume of the solubilized AChE and BChE (i.e. 20,000g supernatant) was mixed with 5µl

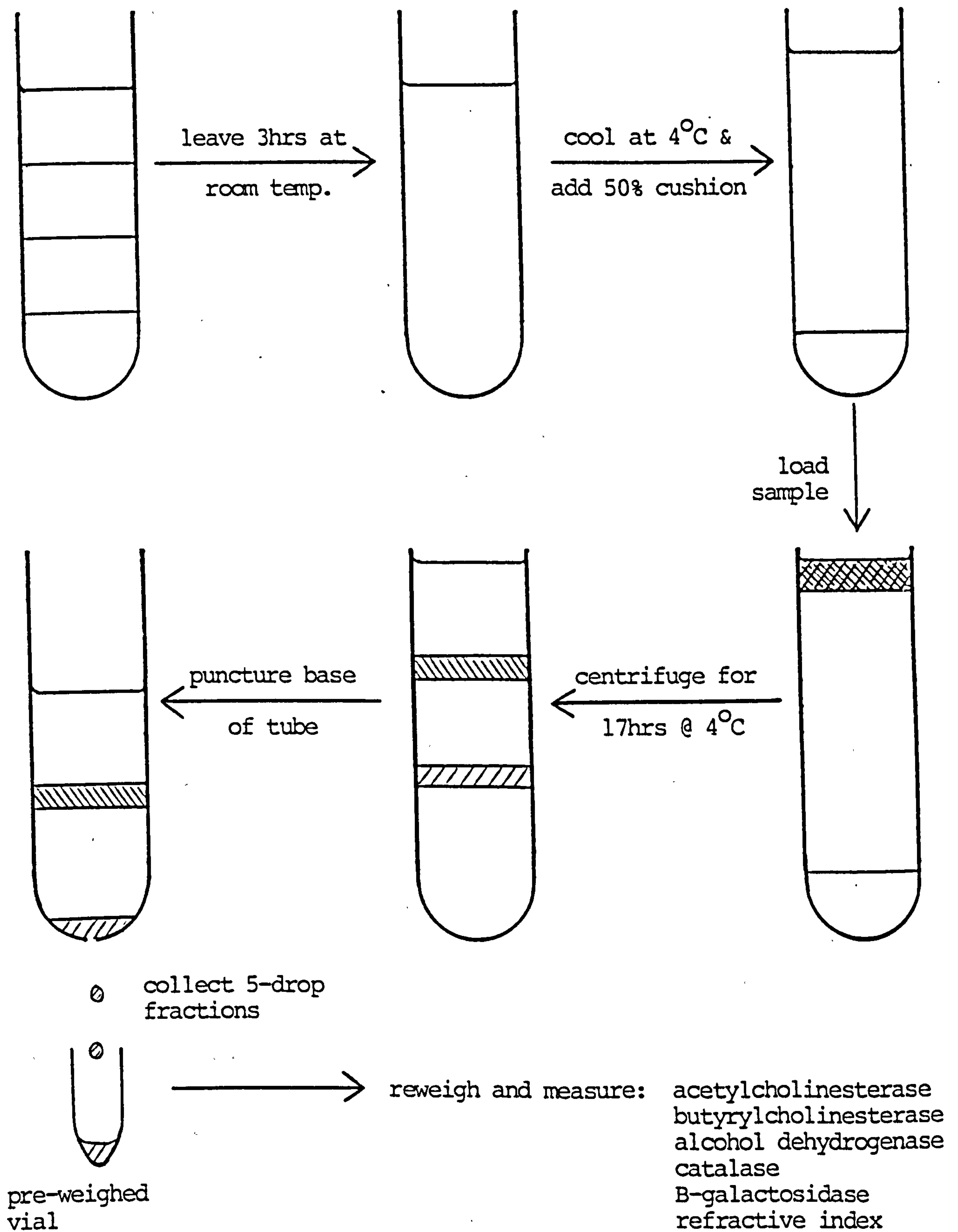


Figure 19. Diagrammatic illustration of the density gradient centrifugation procedures used to separate molecular forms of cholinesterases. Although the gradient shown is a 5-20% plus 50% sucrose "cushion" gradient similar techniques were used for the formation of the 10-40% gradients (see text).



of each of three marker enzymes of known sedimentation velocities. The marker enzymes used were alcohol dehydrogenase (stock concentration,  $12 \times 10^3$  Units/litre; sedimentation velocity, 4.8 Svedberg (S) units), catalase ( $25 \times 10^6$  U/L; 10.4S) and  $\beta$ -galactosidase ( $100 \times 10^3$  U/L; 16.0S). The mixture of sample and markers was then layered on top of the gradient, resulting in a total volume of 5.0ml, ready for centrifugation.

The gradients were centrifuged in a Beckman L2-75B ultracentrifuge using a Beckman SW65Ti swing-out rotor. The conditions of centrifugation were 17 hours at  $4^\circ\text{C}$  at a speed of 37,500rpm (140,000gmax) for the 5-20% plus 50% sucrose cushion gradient, or 55,000rpm (300,000gmax) for the 10-40% gradient. After completion of centrifugation, the centrifuge tubes were removed from the rotor buckets and stored on ice prior to fractionation. Fractionation was performed by piercing the bottom of the centrifuge tube with a 21 gauge hypodermic needle and collecting 5 drop fractions, which resulted in the collection of approximately 35 fractions per gradient. After fractionation the bottom of each tube was sealed with "Parafilm" and 0.5ml of homogenising medium was added to the tube which was then vortexed vigorously. The medium was then poured into a vial for subsequent assay for AChE and BChE activity to determine whether or not any enzyme had pelleted out or adhered to the side of the tube.

Since the plot of activity versus fraction number makes the incorrect assumption that each fraction is of identical size, it is necessary, for accurate quantitation of the peaks of activity recovered from the gradient, that an allowance be made for differences in fraction size. Therefore, the fractions were collected in pre-weighed vials which were re-weighed after fractionation. After the enzyme assays had been performed the refractive indices of 25 $\mu$ l aliquots of the fractions were recorded using a Bellingham and Stanley (London, England) pocket refractometer and from the refractive index the density of each fraction was determined using conversion tables (Griffiths, 1979). Since the density and weight of each fraction is known the volume of each fraction can therefore be calculated and the activity of each gradient fraction can then be plotted versus gradient volume. In addition, since activity is expressed per ml of fraction and the abscissa is in units of volume, the area of each peak can be expressed conveniently in units of activity.

#### d) Enzyme assays

Cholinesterase assays were mostly performed on the same day that the gradient was fractionated. Where this was not possible, fractions were



stored at  $-20^{\circ}\text{C}$  for a maximum of 4 days. The sedimentation velocity markers were all assayed within 2 weeks of storage.

AChE and BChE were assayed using the colorimetric assay procedure of Ellman et al (1961) - see chapter 2, section II.6 - as modified by Bonham et al (1981). In the present experiments the rate of increase in absorbance at a wavelength of 412nm and at  $37^{\circ}\text{C}$  was followed using a Cobas-Bio centrifugal fast analyser. From each gradient fraction, separate 20 $\mu\text{l}$  aliquots were removed and assayed for each cholinesterase activity in 0.1M phosphate buffer, pH 8.0. AChE activity was assayed in the presence of 14 $\mu\text{M}$  ethopropazine (an inhibitor of BChE) using  $0.5 \times 10^{-3}\text{M}$  acetyl- $\beta$ -methylthiocholine as substrate and BChE was assayed in the absence of inhibitors using  $0.5 \times 10^{-3}\text{M}$  butyrylthiocholine as substrate. Under these conditions AChE produced an artefactual interference in the BChE assay of approximately 0.35% (i.e. 100 units of AChE activity produced an artefactual activity in the BChE assay equivalent to 0.35 units of BChE) whilst BChE showed a similar level (0.45%) of cross-reactivity in the AChE assay.

AChE and BChE standards (derived from human erythrocytes and plasma respectively) were included in each batch of assays. At a level of 2.5nmol/min the AChE standard had an inter-batch coefficient of variation of 2.8% ( $n = 65$ ) whilst the BChE standard had a coefficient of variation of 3.3% ( $n = 58$ ) at a level of 11.5nmol/min. Thus, the assay conditions are highly reproducible.

All three marker enzymes were assayed spectrophotometrically and briefly the procedures were as follows: i), alcohol dehydrogenase was measured by incubating 10 $\mu\text{l}$  aliquots of each fraction in a medium containing 1.5ml 0.1M Tris-HCl buffer, pH 8.8, 0.5ml 2M ethanol and 1.0ml  $25 \times 10^{-3}\text{M}$  nicotinamide adenine dinucleotide (NAD) and following the rate of reduction of NAD to NADH at a wavelength of 340nm (Valee and Hoch, 1955); ii), catalase activity was assayed by incubating 5 $\mu\text{l}$  aliquots of each fraction in 1.0ml of 0.05M phosphate buffer, pH 7.0 containing 0.2% v/v hydrogen peroxide and the rate of disappearance of hydrogen peroxide measured at 240nm (Beers and Sizer, 1952) and iii),  $\beta$ -galactosidase was estimated by incubating 10 $\mu\text{l}$  aliquots of each fraction in 1.0ml of a medium consisting of 0.4ml distilled water, 0.33ml of 0.3M phosphate buffer, pH 7.3, 0.1ml of 1.0M mercaptoethanol and 0.17ml of 0.014M O-nitro-phenyl- $\beta$ -D-galactopyranoside (ONPG) and following the increase in absorbance resulting from the hydrolysis of ONPG at 405nm (Craven et al, 1965).



#### e) Quantitation of peaks

Enzyme activity was plotted versus gradient volume and the area under which peak was determined using a Videoplan image analysing computer (Kontron, Zurich, Switzerland) by outlining the peak with the computer pen. Each peak was measured on at least four different occasions and the coefficient of variation between successive determinations was generally less than 4% for the larger peaks and less than 10% for the smaller peaks.

#### f) Molecular forms nomenclature

In the present study the nomenclature of Bon et al (1979) will be used to describe the different molecular forms of AChE and BChE (chapter 1, section II.9). Thus, the forms of AChE and BChE with a sedimentation velocities of approximately 3.5S are designated  $G_1$  and those in the region of 5 to 6.5S are called  $G_2$ . The  $G_4$  form (see also section III.2.b.i) describes the forms of AChE and BChE with sedimentation velocities of 8.5-10.5S and 9.5-11.5S respectively and the molecular form of AChE with a sedimentation velocity of approximately 16S is designated  $A_{12}$ .

#### g) Statistical analysis

Comparisons between groups were made by determining whether or not the ratio of variances of the two groups was significantly different (F-test) and calculating the value of Student's  $t$  accordingly (Bailey, 1964). Statistics were not performed on groups with a sample size of three or less.

### III. RESULTS

#### 1. Preliminary experiments.

##### a) Linearity of the gradients

Figure 20 shows the profile of each type of gradient i.e. 5-20% w/v plus 50% w/v sucrose or 10-40% w/w sucrose with both the percentage sucrose (obtained from the refractive indices of the fractions) and the positions of marker proteins of known sedimentation velocities (S values) plotted as a function of the gradient volume.

Both gradients are essentially linear with respect to sucrose concentration above a gradient volume of approximately 1.5ml. The non-linearity in the case of the 5-20% plus 50% w/v sucrose gradient is due to the 50% cushion, whilst the non-linearity of the 10-40% w/w gradient is probably due to the relatively high viscosity of the lower 40% w/w sucrose, resulting in a lower rate of diffusion of this layer and causing the shape of the gradient to deviate from linearity.

The profile obtained when sedimentation velocity is plotted as a function of gradient volume is similar to that observed when sucrose concentration is plotted as a function of gradient volume. Thus, in both types of gradient, the relative linearity of the region between catalase and alcohol dehydrogenase, and between alcohol dehydrogenase and the top of the gradient means that the S value of any peak sedimenting slower than catalase (i.e. between the top of the gradient and catalase) can be calculated in relation to these markers. However, the non-linear nature of both gradients in the region of the  $\beta$ -galactosidase marker means that the S values of molecular forms sedimenting in this region cannot be accurately determined by extrapolation using alcohol dehydrogenase, catalase and  $\beta$ -galactosidase as internal markers. However, since the molecular forms that were detected in this region all sedimented within approximately 250 $\mu$ l of the  $\beta$ -galactosidase marker, they were assigned an approximate S value of 16S.

##### b) Stability of molecular forms of AChE and BChE postmortem

Representative analyses of one mouse brain analysed immediately after death and one mouse brain analysed after a postmortem interval of 31 hours are shown in figure 21. The two other brains in each group produced essentially identical patterns to those shown here. The



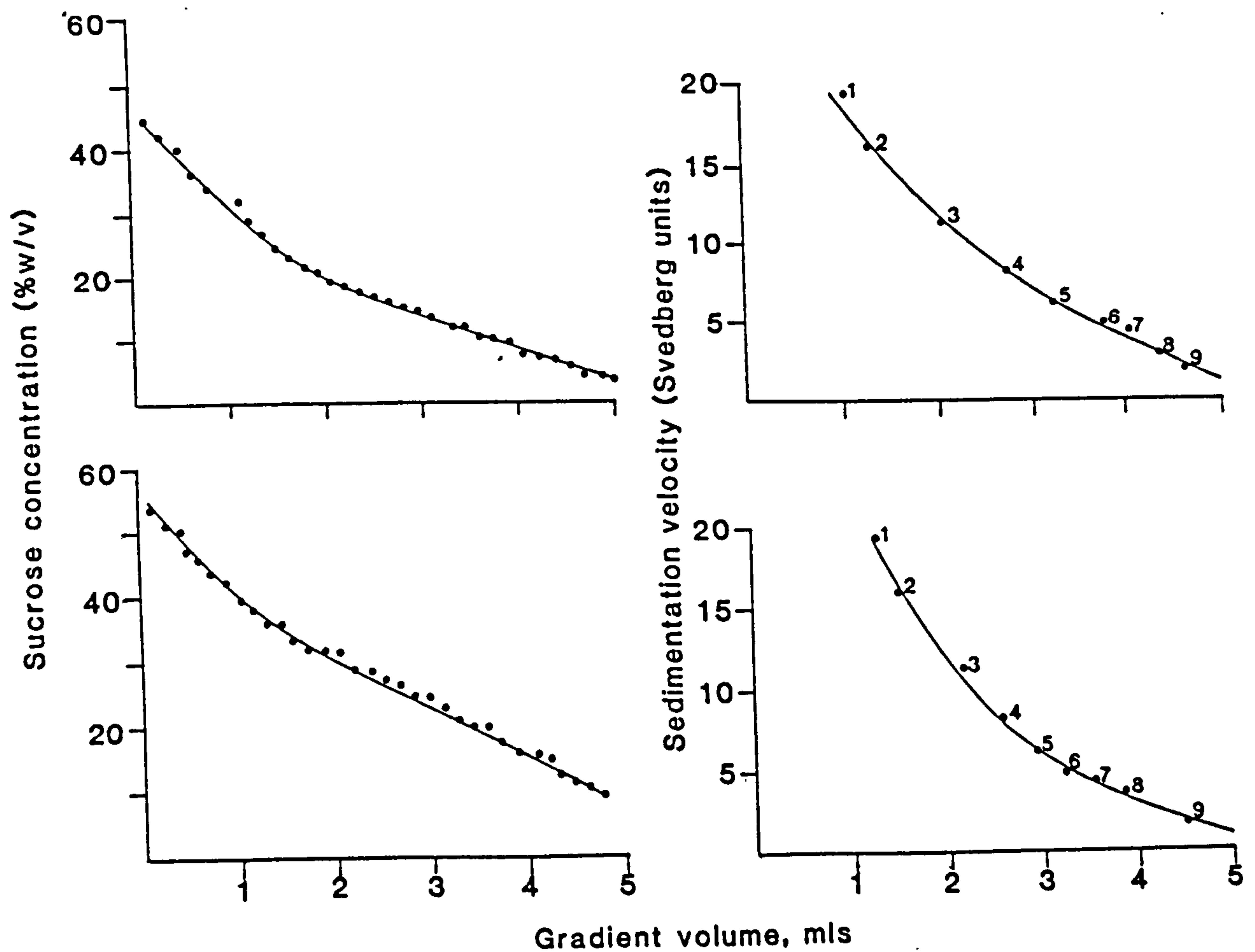


Figure 20. Figures illustrating the profiles after centrifugation of the two types of gradients used in the present study (upper figures, 5-20% plus 50% w/v sucrose gradient; lower figures, 10-40% w/w sucrose gradient). Figures on the left show the sucrose concentration (calculated from the refractive index) at various points of the gradient and those on the right show the positions at which various marker proteins sedimented in the same gradient. Marker proteins and their sedimentation velocities are: 1, thyroglobulin (19.4S); 2,  $\beta$ -galactosidase (16.0S); 3, catalase (11.4S); 4, aldolase (8.3S); 5, alkaline phosphatase (6.1S); 6, alcohol dehydrogenase (4.8S); 7, haemoglobin (4.2S); 8, ovalbumin (3.6S) and 9, cytochrome c (1.7S).

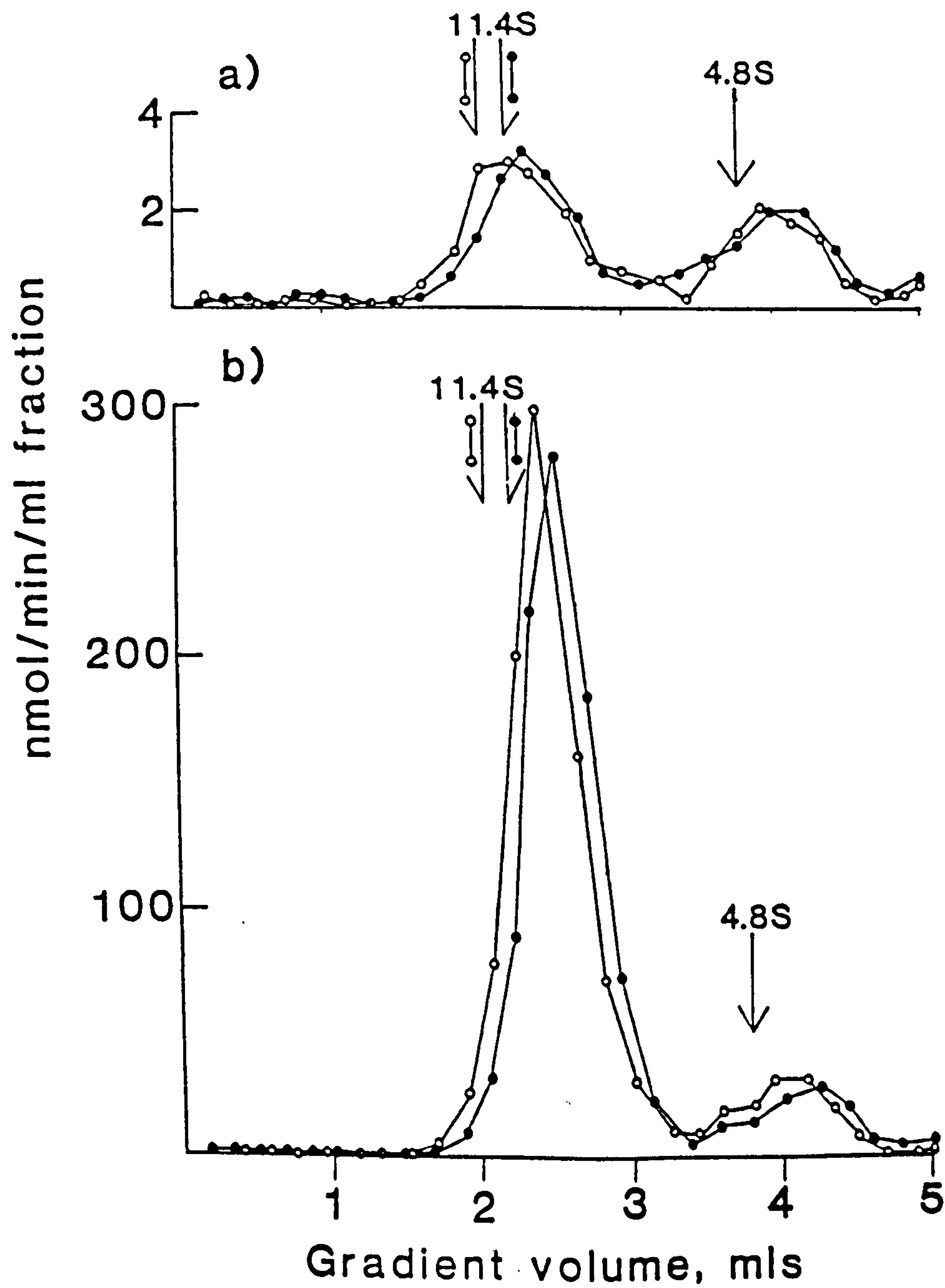


Figure 21. Figure illustrating the molecular forms of mouse brain BChE (a) and AChE (b) analysed immediately after sacrifice (●—●) or after a postmortem delay of 31 hours (○—○). Load = 200 $\mu$ l solubilised enzyme (derived from the equivalent of 20mg wet weight of tissue). Sedimentation velocity markers = alcohol dehydrogenase (4.8S) and catalase (11.4S).



activities of the molecular forms of AChE were:  $G_1$ , analysed "fresh",  $0.89 \pm 10 \mu\text{mol}/\text{min}/\text{g}$  wet weight of tissue; analysed "postmortem",  $1.08 \pm 0.17 \mu\text{mol}/\text{min}$ ;  $G_4$ , fresh,  $7.22 \pm 1.10 \mu\text{mol}/\text{min}$ ; postmortem,  $7.50 \pm 0.66 \mu\text{mol}/\text{min}$ . For BChE the activities (note different units) were:  $G_1$ , fresh,  $72 \pm 13 \text{nmol}/\text{min}$ ; postmortem,  $67 \pm 8 \text{nmol}/\text{min}$ ;  $G_4$ , fresh,  $105 \pm 13 \text{nmol}/\text{min}$ ; postmortem,  $117 \pm 13 \text{nmol}/\text{min}$ . These results clearly show that, in mice, both AChE and BChE molecular forms are stable postmortem.

c) Effects of protease inhibitors on the extraction of molecular forms of cholinesterases in human neocortex.

Figure 22 and table 16 show an analysis of the data for the molecular forms of AChE and BChE obtained in the absence and presence of protease inhibitors. The patterns of distribution and the levels of activity of each peak are very similar for the extractions carried out in the presence and absence of protease inhibitors and suggests that endogenous proteases have no significant effect on the S value, distribution or relative activities of the different molecular forms of human neocortical AChE or BChE.

d) Stability of human brain cholinesterases upon storage of extracts.

Figure 23 shows the distribution of molecular forms obtained from human temporal cortex and caudate nucleus analysed immediately after extraction and after storage for 4 weeks at  $-20^\circ\text{C}$ . In the freshly analysed temporal cortex and caudate nucleus extracts, the majority of the AChE activity was present as a major (60% and 95% of the total activity respectively)  $G_4$  peak of AChE with a less abundant  $G_1$  peak accounting for the remaining activity in both types of extract. Whilst BChE activity was not determined in the caudate nucleus extracts, in the temporal cortex the  $G_4$  and  $G_1$  forms of BChE constituted 41 and 59% of the recovered activity respectively. After 4 weeks storage at  $-20^\circ\text{C}$  the activity of the caudate nucleus extract fell from 2156 to 1675 nmol/min/ml although the activities of both AChE and BChE in the temporal cortex extracts remained very stable (AChE, 50.8 and 50.9 nmol/min/ml and BChE, 25.8 and 26.5 nmol/min/ml before and after storage respectively). With respect to the molecular forms, major changes in the distribution of AChE were observed whilst, in contrast, BChE was much more stable. Hence, in both the caudate nucleus and temporal cortex extracts there was a striking decrease in the amount of  $G_4$  form of AChE. Thus, after storage the proportion of the total activity due to the  $G_4$  form fell to 40% and

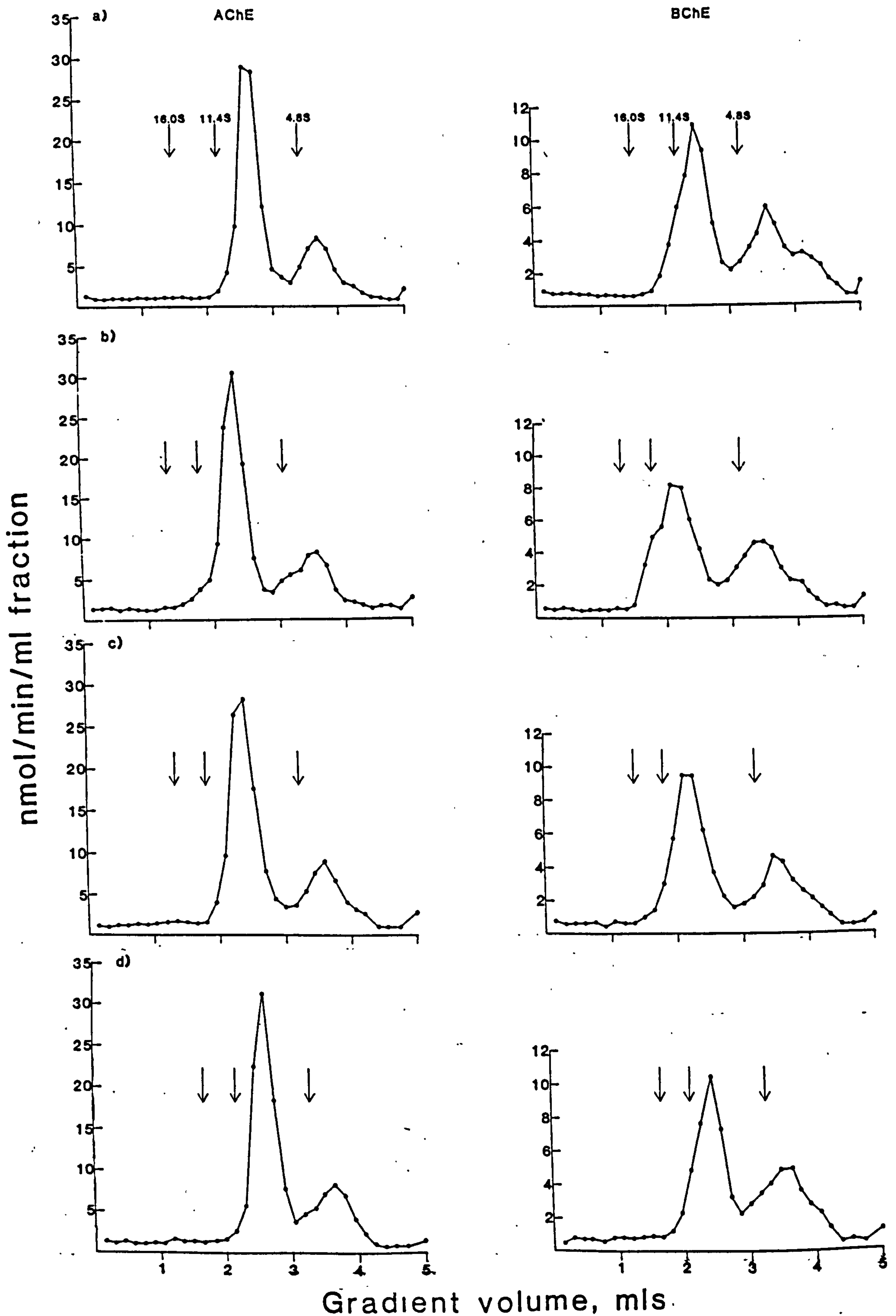


Figure 22. Extraction of molecular forms of AChE (left hand side) and BChE (right hand side) in human temporal cortex in relation to proteolysis. Extractions carried out in homogenising medium: a) in the absence of protease inhibitors; b) plus EDTA; c) plus bacitracin, benzamidine hydrochloride, N-ethylmaleimide and pepstatin d) plus aprotinin, bacitracin, benzamidine hydrochloride, N-ethylmaleimide, leupeptin, pepstatin and trypsin inhibitors (chick, lima bean and soya bean). Load = 400 $\mu$ l solubilised enzyme (derived from the equivalent of 40mg wet weight of tissue). Sedimentation velocity markers = alcohol dehydrogenase (4.8S), catalase (11.4S) and  $\beta$ -galactosidase (16.0S).



Table 16

The extraction of molecular forms of acetylcholinesterase and butyrylcholinesterase in the presence and absence of protease inhibitors

	Acetylcholinesterase				Butyrylcholinesterase			
	G <sub>4</sub> form		G <sub>1</sub> form		G <sub>4</sub> form		G <sub>1</sub> form	
	<sup>b</sup> S	<sup>c</sup> mU	S	mU	S	mU	S	mU
medium 1. <sup>a</sup>	8.43	11.79	3.42	4.19	9.17	5.38	3.75	3.92
medium 2. <sup>a</sup>	8.61	12.38	3.68	4.69	9.75	5.23	3.97	3.41
medium 3. <sup>a</sup>	8.97	13.29	3.79	4.69	9.75	5.54	4.13	3.31
medium 4. <sup>a</sup>	8.79	11.91	3.81	4.48	9.49	5.05	4.02	3.67

<sup>a</sup> see also Materials and Methods.

<sup>b</sup> S value = sedimentation velocity (Svedberg units)

<sup>c</sup> mU = nmol/min

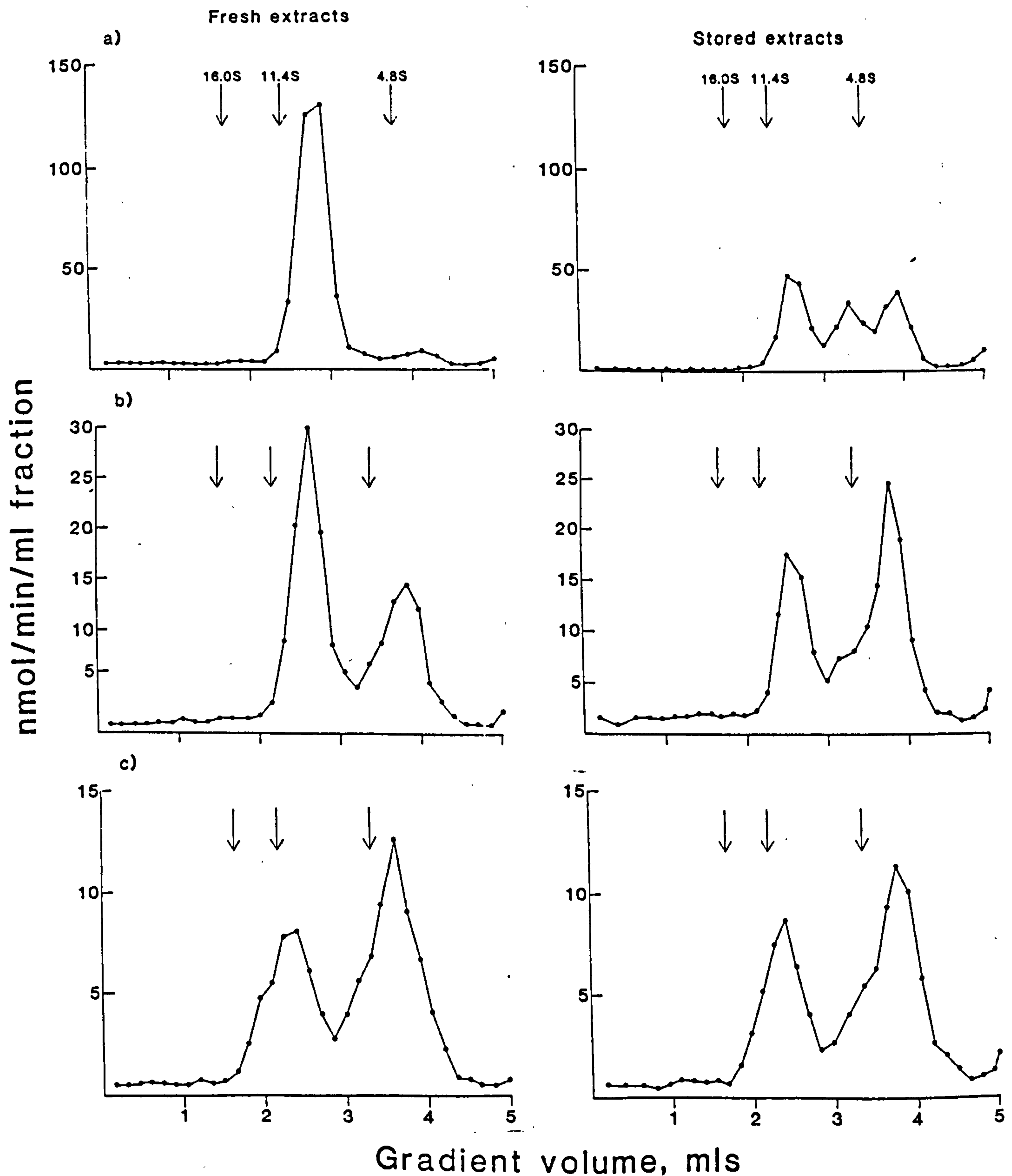


Figure 23. Figure showing the effects of storage on molecular forms of human temporal cortex and caudate nucleus. Figures on the left illustrate analyses of fresh extracts and figures on the right show analyses of the same extracts stored at  $-20^{\circ}\text{C}$  for 4 weeks.

a) Caudate nucleus AChE, b) temporal cortex AChE and c) temporal cortex BChE. Load =  $40\mu\text{l}$  of caudate nucleus extract and  $400\mu\text{l}$  of temporal cortex extract (derived from the equivalent of 4 and 40mg wet weight of tissue respectively). Sedimentation velocity markers = alcohol dehydrogenase (4.8S), catalase (11.4S) and  $\beta$ -galactosidase (16.0S).



64% of that observed in the fresh caudate nucleus and temporal cortex extracts respectively. This reduction in  $G_4$  activity was accompanied in both extracts by an increase in activity of the  $G_1$  form and the appearance in the caudate nucleus extract of a discrete peak of  $G_2$  activity. Furthermore, although a discrete  $G_2$  peak was not observed in the stored temporal cortex extracts, a shoulder of faster sedimenting activity associated with the  $G_1$  peak is probably due to the  $G_2$  form. In contrast, the molecular forms of BChE appear to be relatively unaffected by storage at  $-20^{\circ}\text{C}$  and the molecular forms observed after this period had a similar distribution of both the  $G_4$  and  $G_1$  forms observed in the freshly analysed extracts.

## 2. Distribution of molecular forms in the normal human central nervous system

### a) Molecular forms of AChE and BChE in different regions of the human central nervous system

The levels of AChE and BChE activity in various parts of the human CNS are presented in Table 17. These data are consistent with previous observations (Foldes et al, 1962; Domino et al, 1973; Cote and Kremzner, 1983) that the levels of AChE activity vary much more from region to region than the BChE activity. The molecular forms of AChE and BChE are shown in figures 24 and 25. In all regions the extraction of both AChE and BChE from the homogenate was generally greater than 90%. The recovery of activity loaded onto the gradients was of the order of 75% for AChE and slightly less, 70%, for BChE and since no activity was found in medium used to wash out the centrifuge tube after fractionation, the loss of activity is not due to activity pelleting out. An analysis of the data is presented in Table 18.

The data shows that two forms of AChE representing  $G_4$  and  $G_1$  quaternary structures respectively were present in all areas examined. The relative abundance of these forms (ratio  $G_4$  to  $G_1$ ) varies around 13-fold from approximately 1.7 in the temporal cortex to 21 in the caudate nucleus. In contrast, whilst the analogous  $G_4$  and  $G_1$  forms of BChE are also found in all areas examined, their relative abundance (ratio  $G_4/G_1$ ) is much less variable, ranging from 0.9 in the temporal cortex to 4.1 in the CSF, although single substantia nigra and spinal cord samples had ratios of 4.6 and 0.7 respectively.

Table 17

Levels of acetyl- and butyryl-cholinesterase activities<sup>a</sup>  
in different regions of the human central nervous system

<sup>b</sup> Activity of homogenate

	nmol/min/g wet weight				Ratio AChE/ BChE
	AChE	(% caudate)	BChE	(% caudate)	
Caudate nucleus	27930 $\pm$ 3470	(100)	487 $\pm$ 57	(100)	57.4
Parietal cortex	513 $\pm$ 52	(1.8)	247 $\pm$ 62	(50.7)	2.1
Nucleus of Meynert <sup>c</sup>	18800 $\pm$ 12300	(67.3)	N/D		
Temporal cortex	738 $\pm$ 146	(2.6)	364 $\pm$ 87	(74.7)	2.0
Hippocampus	1534 $\pm$ 185	(5.5)	408 $\pm$ 116	(83.8)	3.8
Spinal cord	2046, 2455	(8.0)	348, 367	(73.5)	5.9, 6.7
Substantia nigra	5801, 7185	(23.8)	527, 651	(120.9)	11.0, 11.0
Cerebellum	6063 $\pm$ 1069	(21.7)	717 $\pm$ 10.6	(147.2)	8.5
Fornix	690, 581	(2.3)	200, 304	(51.7)	3.5, 1.9
Cerebrospinal fluid	11.27 $\pm$ 2.58		4.71 $\pm$ 2.43		2.4

<sup>a</sup> Values shown are mean  $\pm$  standard deviation, except for the substantia nigra, fornix and spinal cord where individual values are shown

<sup>b</sup> Activity of the cerebrospinal fluid is expressed as nmol/min/ml

<sup>c</sup> Activity converted to units of g wet weight assuming protein content = 10% wet weight (McGeer and McGeer, 1976; Perry et al, 1977a)

N/D = not detected



Figure 24. Diagram showing the representative distributions of molecular forms of AChE in various regions of the human central nervous system. Although different loads of the different types of tissue were applied to the gradients, the ordinate axes have been adjusted so that the activities shown are all derived from an equivalent amount (1g wet weight) of tissue. Actual amounts of extracts loaded were derived from the equivalent of 280 $\mu$ g wet weight nucleus of Meynert, 4mg caudate nucleus, 15mg cerebellum and substantia nigra, 25mg of spinal cord and 40mg of parietal and temporal neocortex, fornix and hippocampus. Activity shown for the cerebrospinal fluid are for a load of 1ml (actual load = 0.3ml). Areas shown are: 1, caudate nucleus; 2, parietal cortex; 3, nucleus of Meynert; 4, temporal cortex; 5, hippocampus; 6, spinal cord; 7, substantia nigra; 8, cerebellum; 9, fornix and 10, cerebrospinal fluid. Sedimentation velocity markers = alcohol dehydrogenase (4.8S), catalase (11.4S) and  $\beta$ -galactosidase (16.0S).

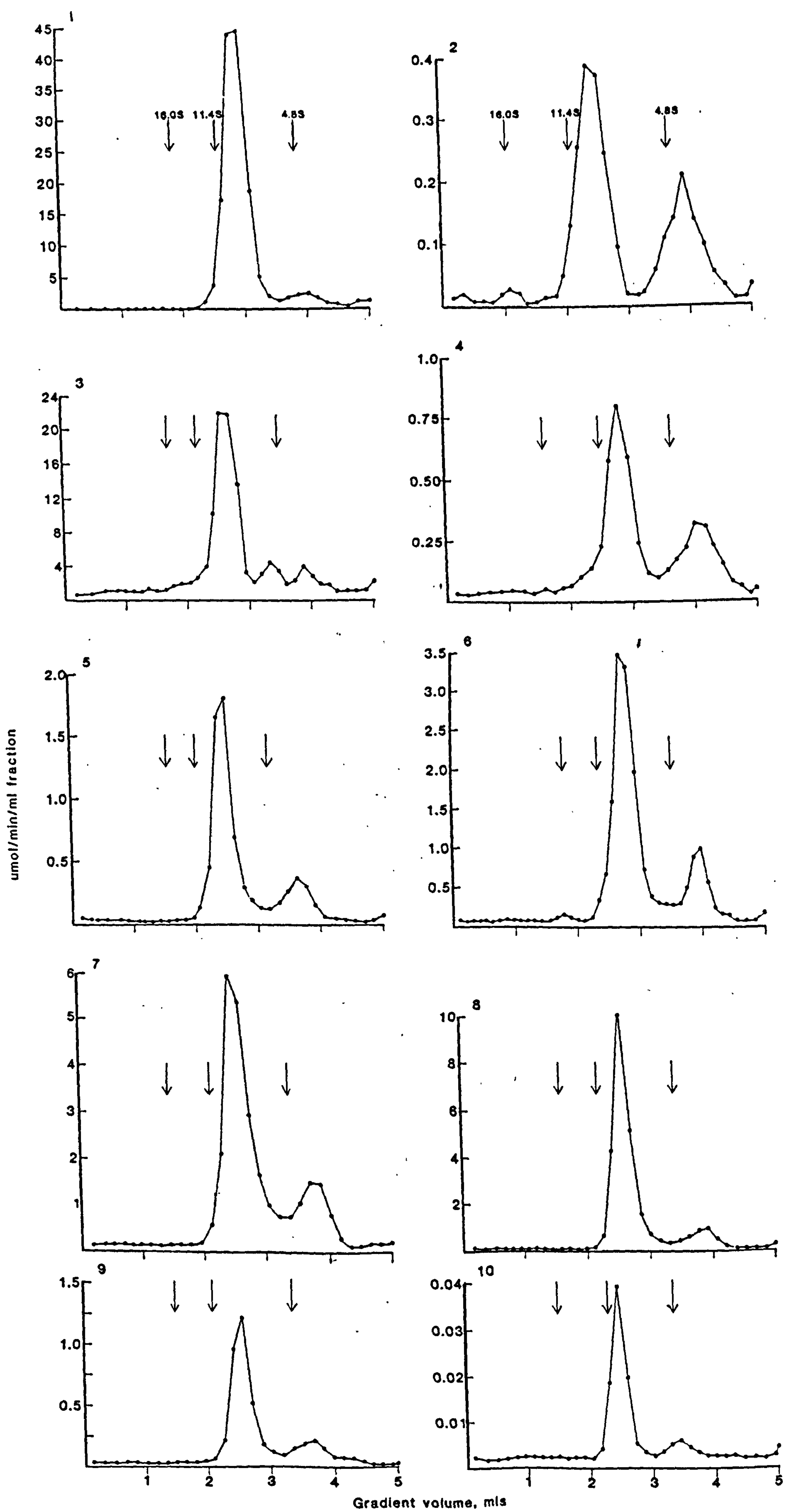
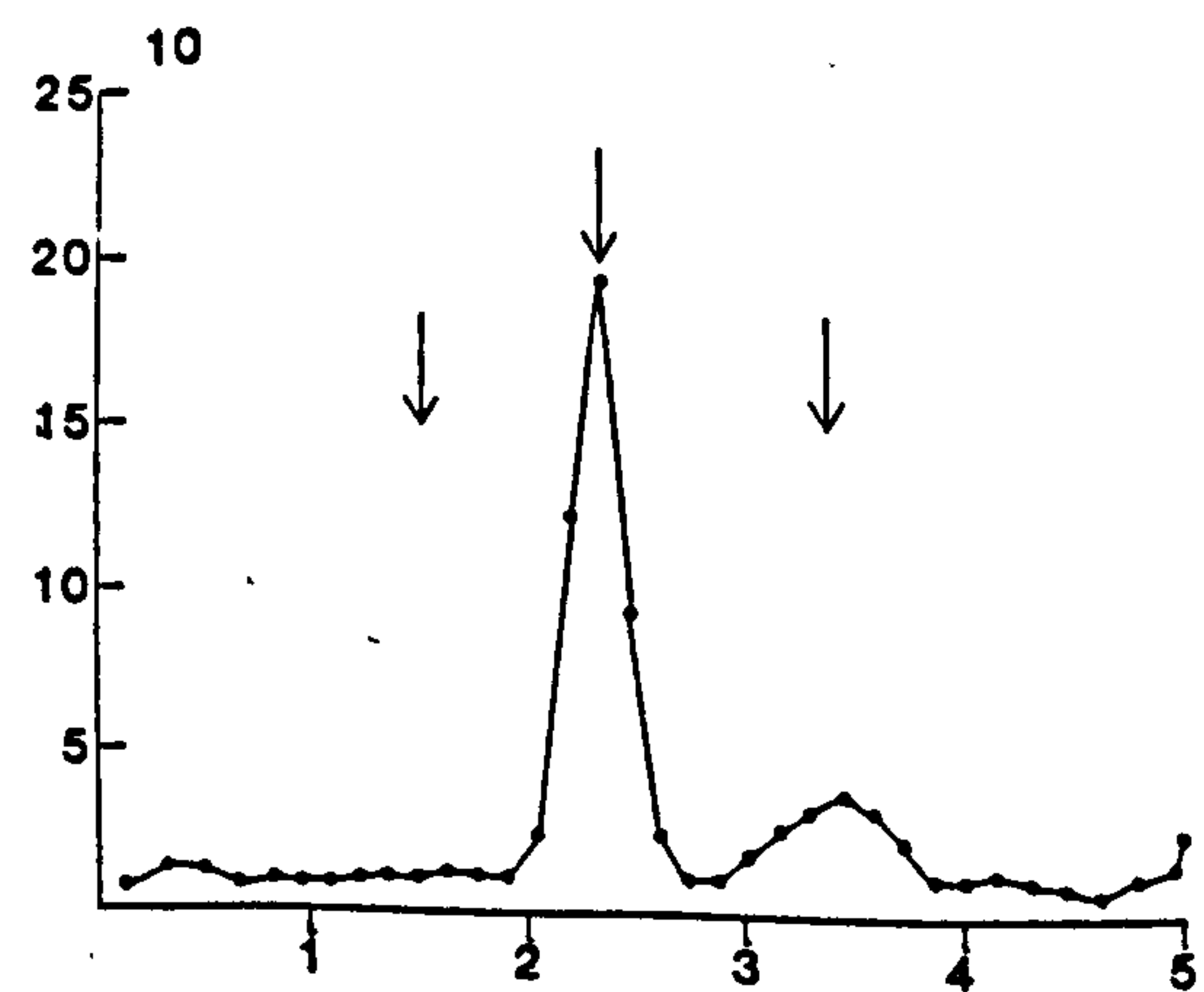
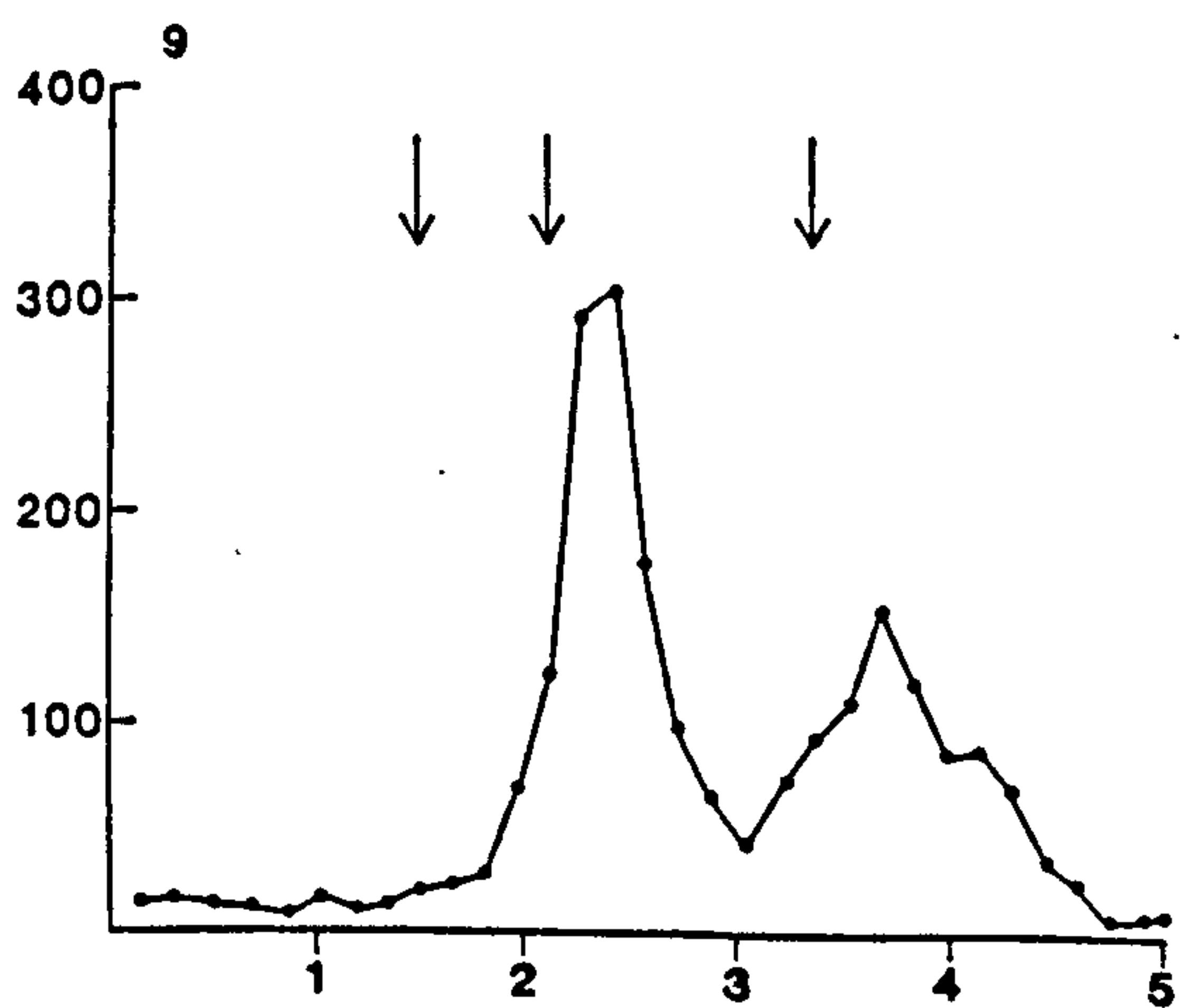
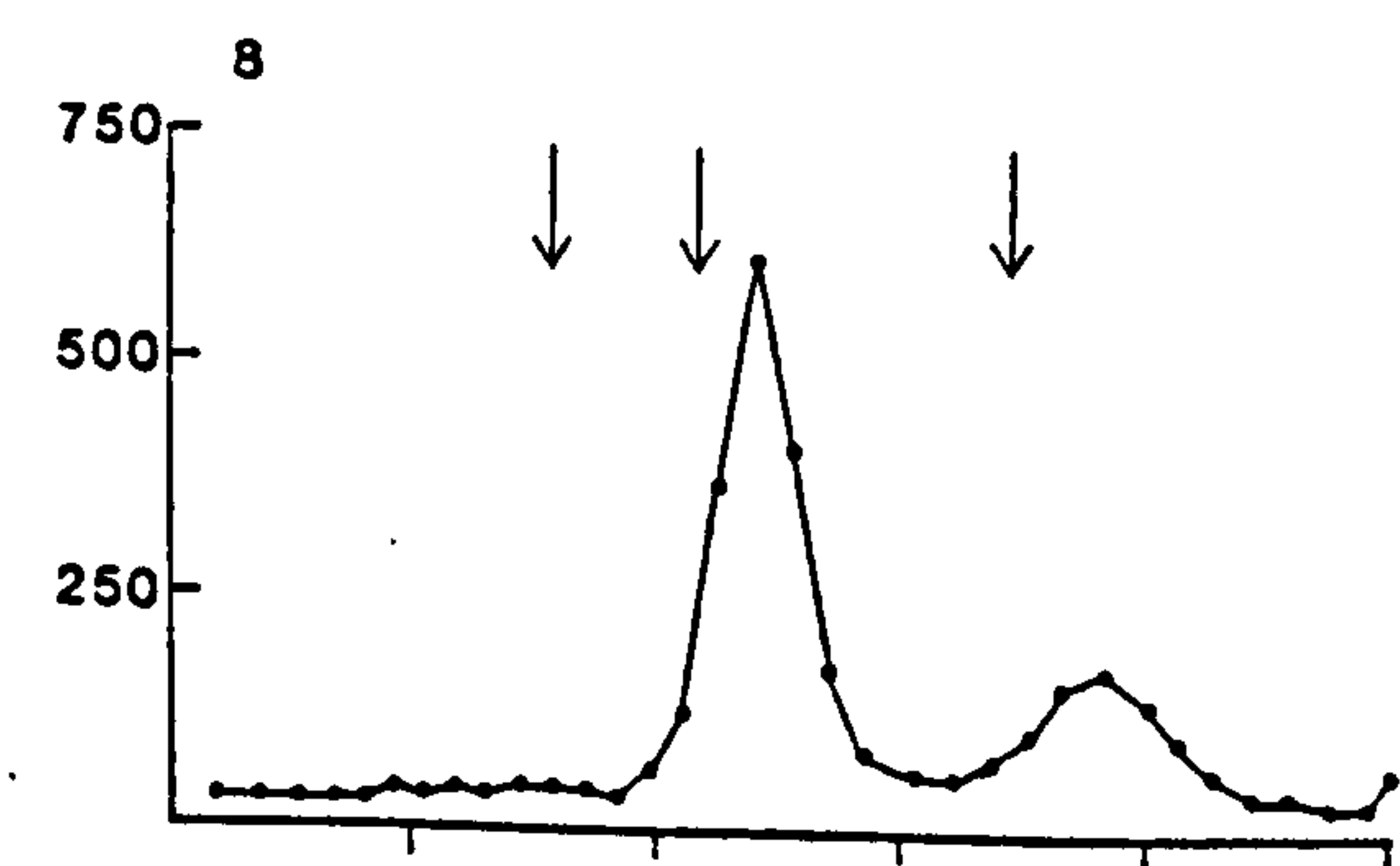
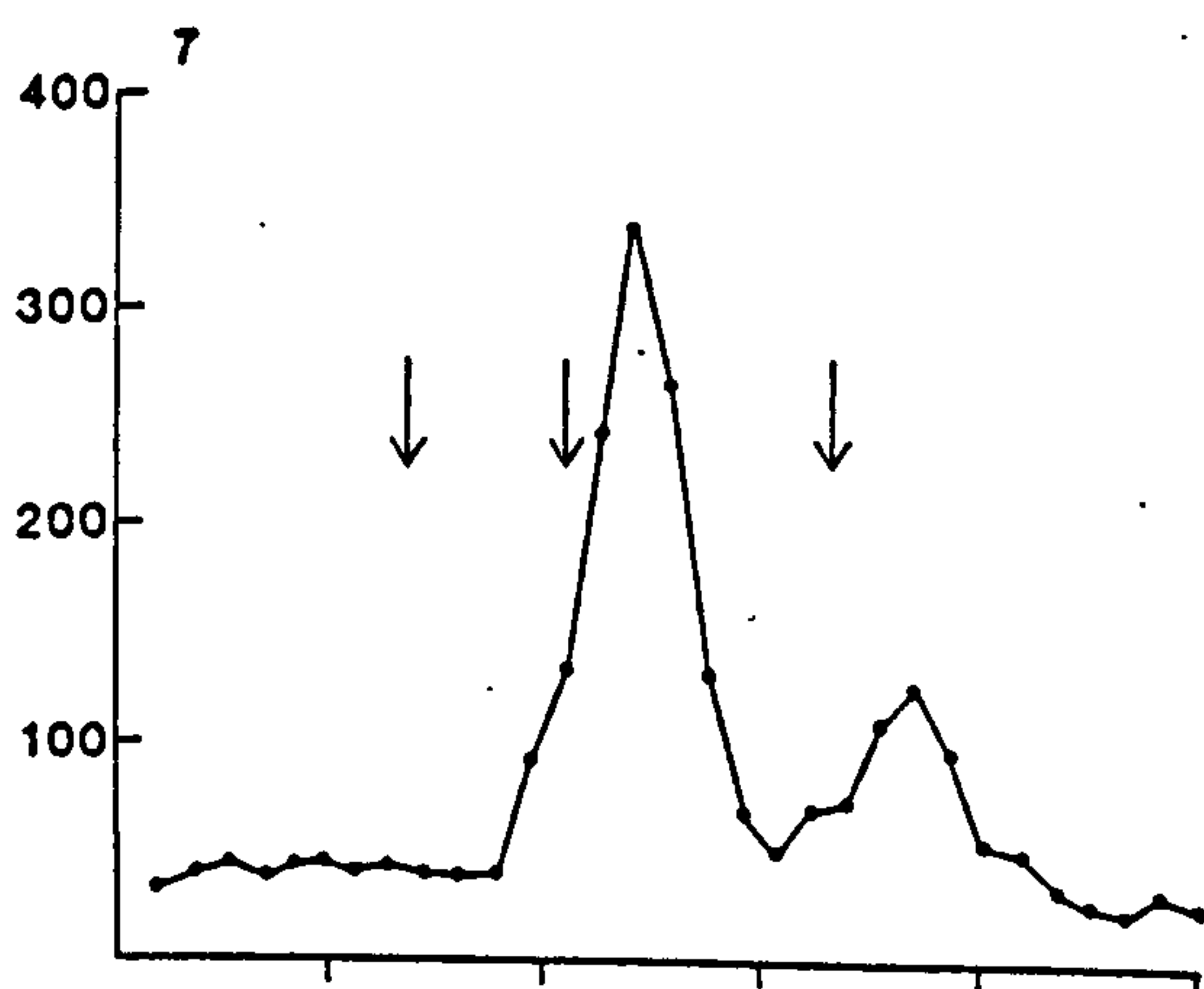
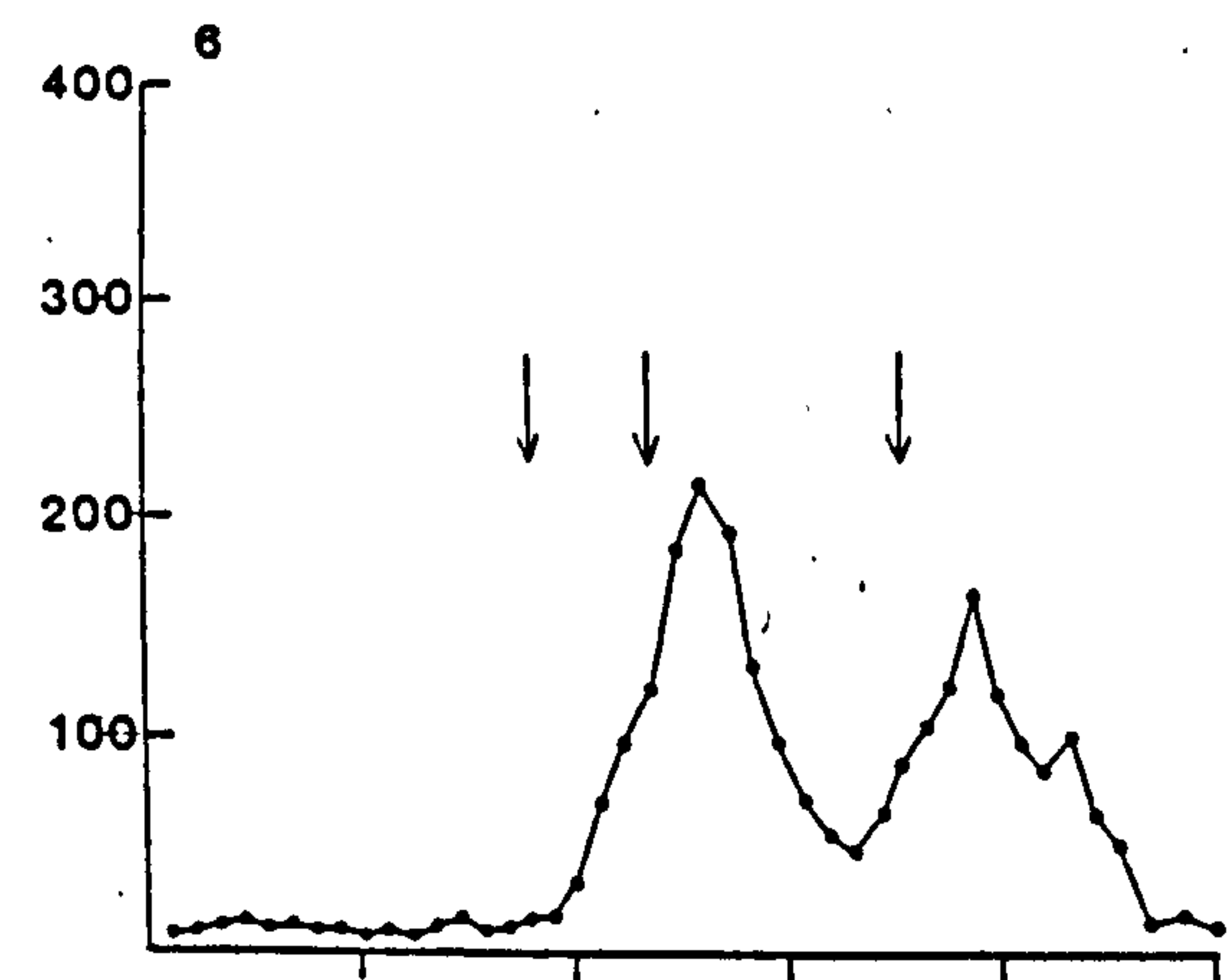
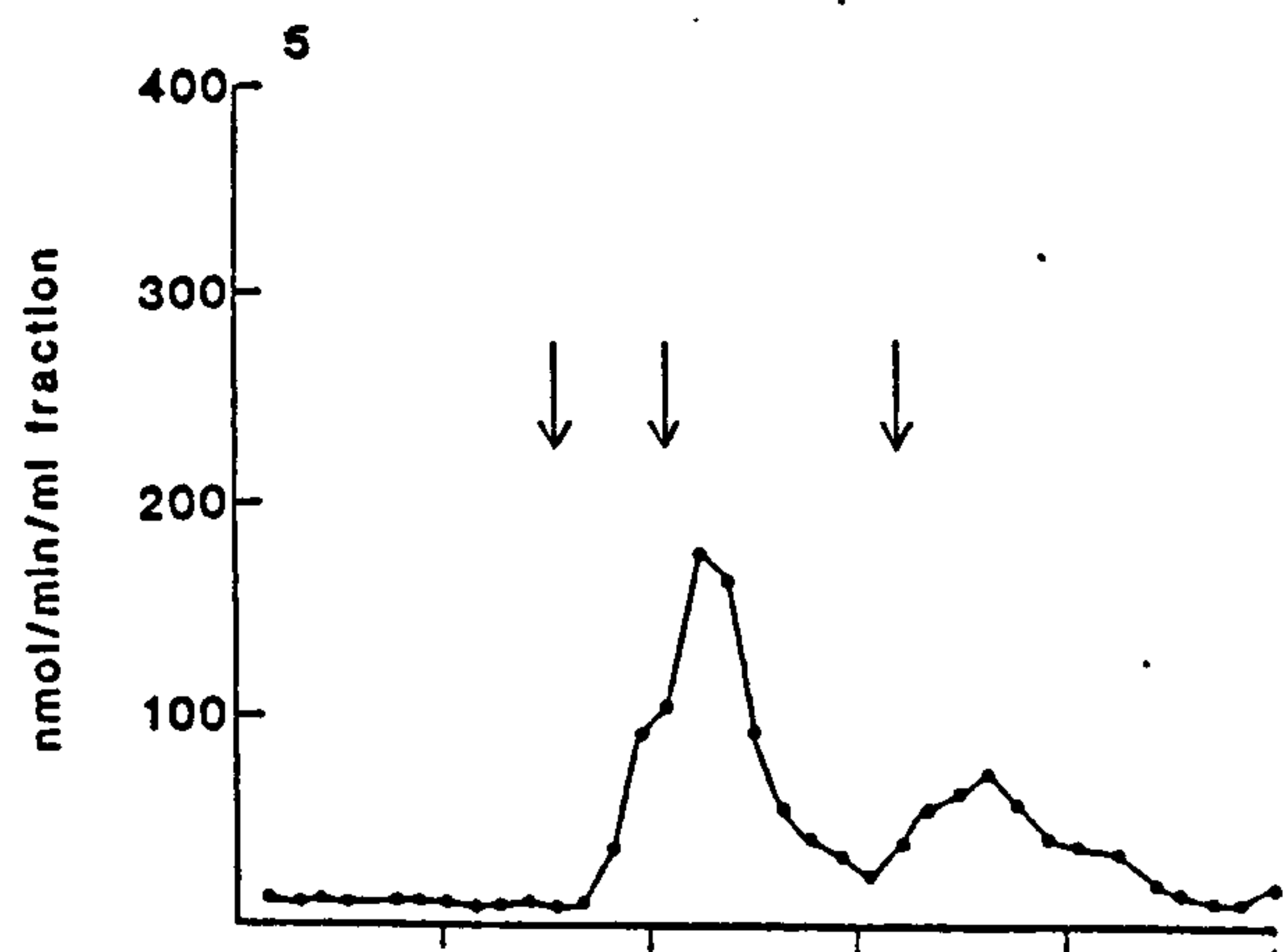
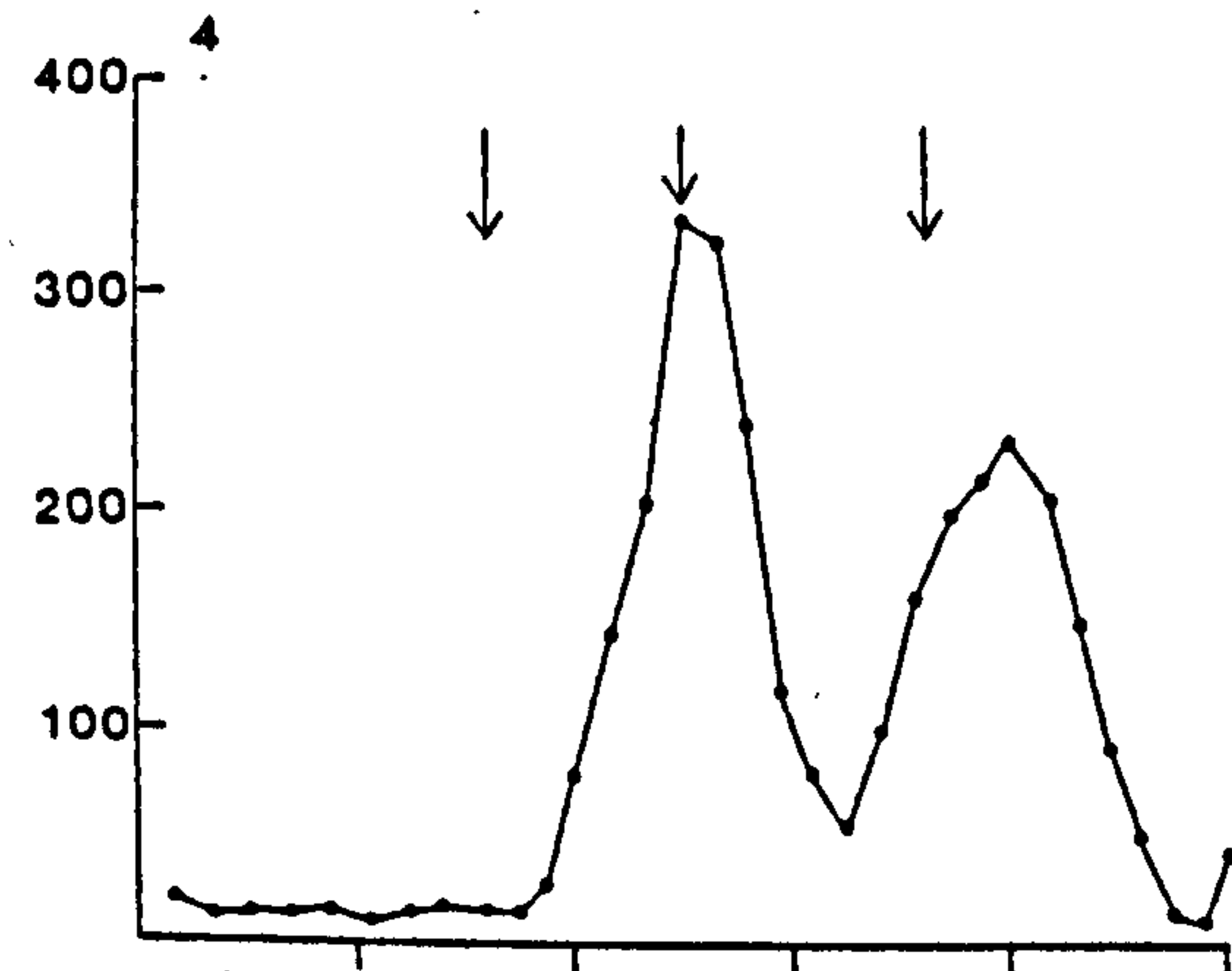
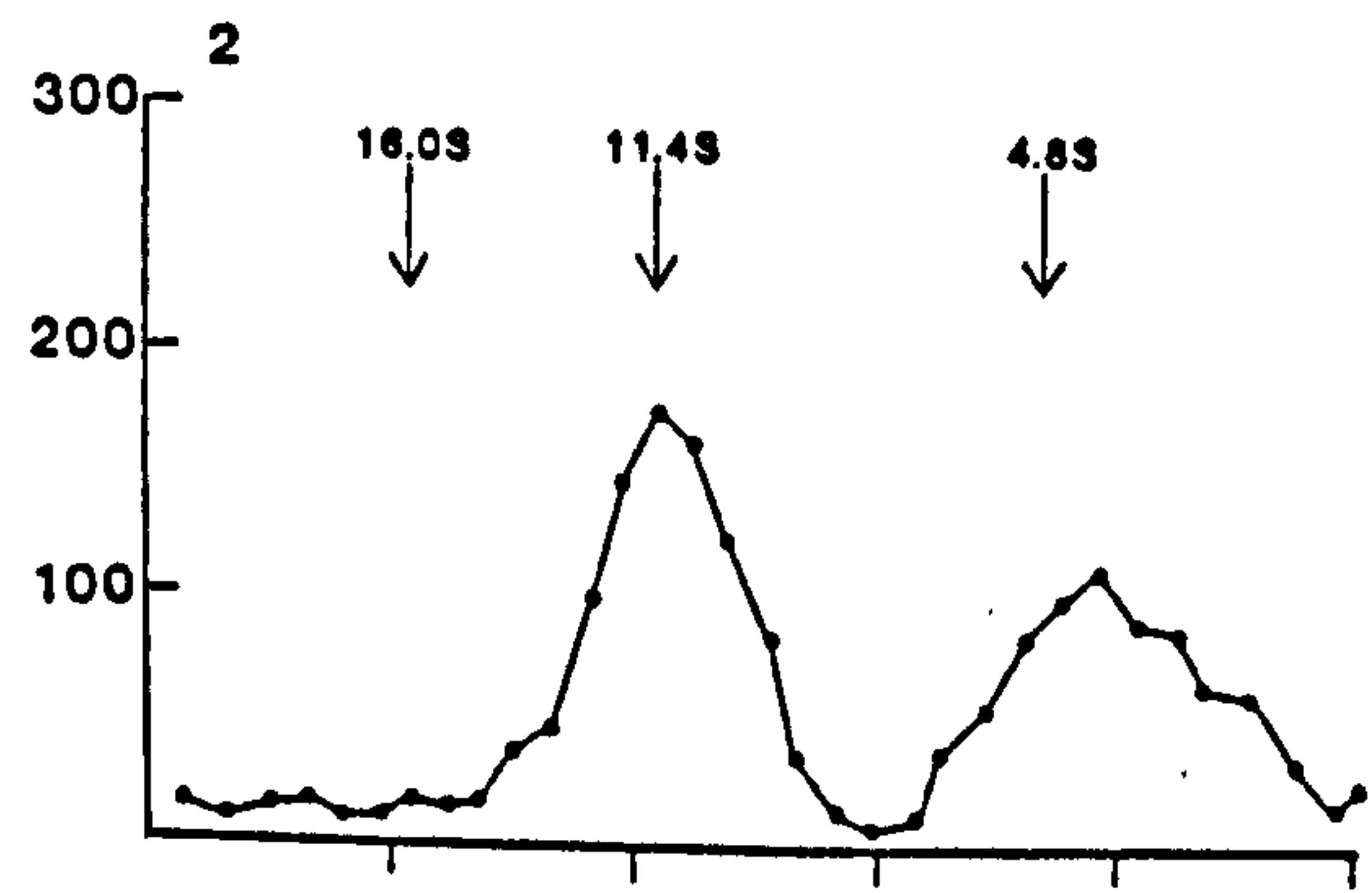
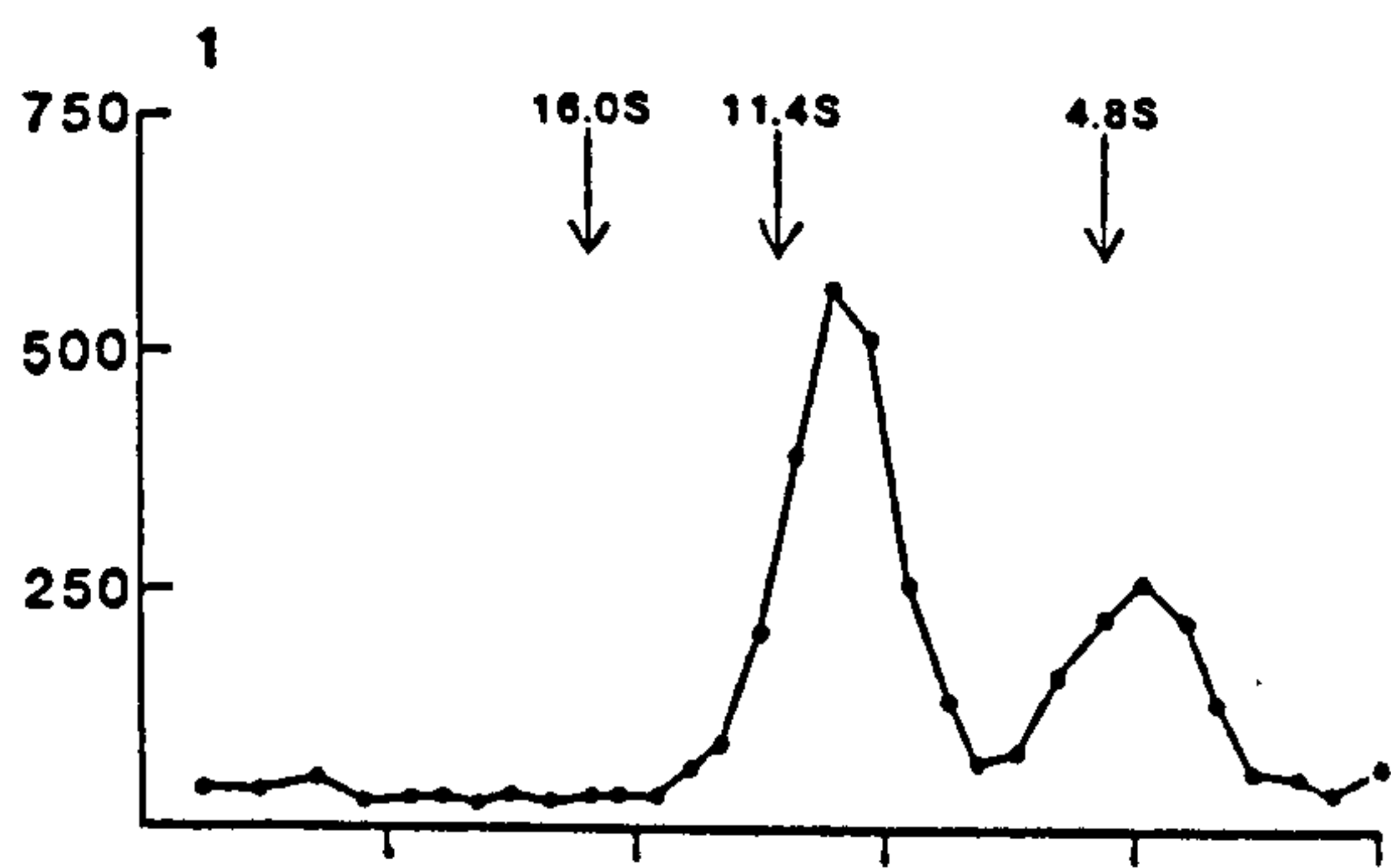




Figure 25. Diagram showing the representative distributions of the molecular forms of BChE in various regions of the human central nervous system. The results illustrated are from the corresponding individual cases shown in figure 24. Ordinate axes have been adjusted so that activities shown are for comparable amounts (1mg wet weight tissue) of tissue (see legend figure 24 for actual loads used). Areas shown are: 1, caudate nucleus; 2, parietal cortex; 4, temporal cortex; 5, hippocampus; 6, spinal cord; 7, substantia nigra; 8, cerebellum; 9, fornix and 10, cerebrospinal fluid (BChE activity not detected in area 3, the nucleus of Meynert). Sedimentation velocity markers = alcohol dehydrogenase (4.8S), catalase (11.4S) and  $\beta$ -galactosidase (16.0S).



Gradient volume, ml



Table 18  
Distribution of acetylcholinesterase and butyrylcholinesterase  
in different regions of the human central nervous system <sup>a</sup>

	<u>ACETYLCHOLINESTERASE</u>						
	$G_1$		$G_4$		$A_{12}$		$G_4:G_1$ ratio
	Amount <sup>b</sup> mU/g	% of total	Amount mU/g	% of total	Amount mU/mg	% of total	
Caudate nucleus	909 ± 161	(4.5)	19352 ± 2427	(95.5)	not detected		21.3
Parietal cortex	119 ± 23	(31.5)	251 ± 25	(66.6)	7.3 ± 3.5	(1.9)	2.11
Nucleus of Meynert	2107 ± 1036	(14.4)	11321 ± 7036	(77.5)	<sup>c</sup> 1179 ± 643	(8.1)	5.37
Temporal cortex	201 ± 19	(37.6)	334 ± 12	(62.4)	not detected		1.66
Hippocampus	160 ± 63	(14.3)	960 ± 217	(85.7)	not detected		6.01
Spinal cord	368, 362	(23.3)	1050, 1370	(76.1)	5.2, 15.6	(0.63)	3.31
Substantia nigra	897, 553	(16.0)	3193, 4932	(84.0)	not detected		6.24
Cerebellum	572 ± 116	(12.9)	3864 ± 458	(87.1)	not detected		6.76
Fornix	83, 85	(18.3)	440, 327	(81.7)	not detected		4.57
Cerebrospinal fluid	1.57 ± 0.38	(11.4)	9.32 ± 1.59	(85.6)	not detected		5.94

<sup>c</sup>G<sub>2</sub> form

	<u>BUTYRYLCHOLINESTERASE</u>				
	$G_1$		$G_4$		$G_4:G_1$ ratio
	Amount <sup>b</sup> mU/g	% of total	Amount mU/g	% of total	
Caudate nucleus	122 ± 4	(31.4)	267 ± 17	(68.6)	2.19
Parietal cortex	95 ± 22	(49.0)	99 ± 27	(51.0)	1.04
Nucleus of Meynert	not detected		not detected		
Temporal cortex	148 ± 29	(54.1)	125 ± 47	(45.9)	0.85
Hippocampus	121 ± 39	(41.2)	172 ± 31	(58.8)	1.43
Spinal cord	157, 122	(53.7)	102, 139	(46.3)	0.90
Substantia nigra	96, 85	(21.8)	278, 389	(78.2)	3.73
Cerebellum	128 ± 55	(30.7)	372 ± 13	(69.3)	2.25
Fornix	50, 88	(38.0)	90, 130	(62.0)	1.64
Cerebrospinal fluid	1.31 ± 0.19	(19.6)	5.37 ± 1.43	(80.4)	4.10

<sup>a</sup> Values shown are mean ± standard deviation, except for the substantia nigra, fornix and spinal cord where individual values are shown

<sup>b</sup> mU = nmol hydrolysed/minute

The only regions in which AChE forms other than  $G_1$  and  $G_4$  were found include the parietal cortex, nucleus of Meynert and spinal cord. Thus, in the parietal cortex, a small amount (1.9%) of the  $A_{12}$  (16S) form was consistently detected yet interestingly was not observed in the temporal cortex. In addition, small amounts (on average 0.6%) of the  $A_{12}$  form were also observed in both samples of spinal cord analysed and although not clearly visible in figure 24 were readily identified when the activity was plotted on a larger scale axis. The only region of the normal CNS in which the  $G_2$  form of AChE was observed was in the nucleus of Meynert where it represented approximately 8% of the total activity recovered off the gradient.

In contrast to AChE, no discrete peaks of activity associated with any other molecular forms of BChE other than the  $G_1$  and  $G_4$  forms were found in any of the areas studied. However, the  $G_1$  and  $G_4$  peaks of BChE were not as well defined as those observed for the corresponding peaks of AChE activity. Thus, in many cases the peaks of BChE activity were not symmetrical and common features were faster and slower sedimenting shoulders of activity associated with the  $G_4$  and  $G_1$  peaks respectively.

#### b) Solubility characteristics of parietal cortex AChE and BChE

##### i) Extractions in the absence and presence of high salt concentrations

Figure 26 shows the high salt solubility characteristics of AChE and BChE found in normal parietal cortex. The majority (93%) of the 10S form of AChE can be extracted in low-salt containing medium and since asymmetric forms are not extracted under low salt conditions this indicates that the 10S form is globular. A small amount of 10S AChE also appears to be extracted by high salt conditions suggesting that this portion might therefore be asymmetric. However, there also appears to be a portion of the 3.5S form of AChE which is extracted in high salt and since no asymmetric form with a sedimentation velocity in this region exists (Massoulie and Bon, 1982), it would appear that small amounts of globular 3.5S and 10S AChE are present as contaminants in the pellet that is subsequently re-extracted under the high salt conditions. The quaternary structures of AChE corresponding to globular forms with sedimentation coefficients of 10S and 3.5S are  $G_4$  and  $G_1$  respectively.

Similarly, the majority of both the 11.5S and 3.5S forms of BChE were also extracted in low salt medium and therefore, since molecular forms of BChE and AChE have similar solubility characteristics (Allemand



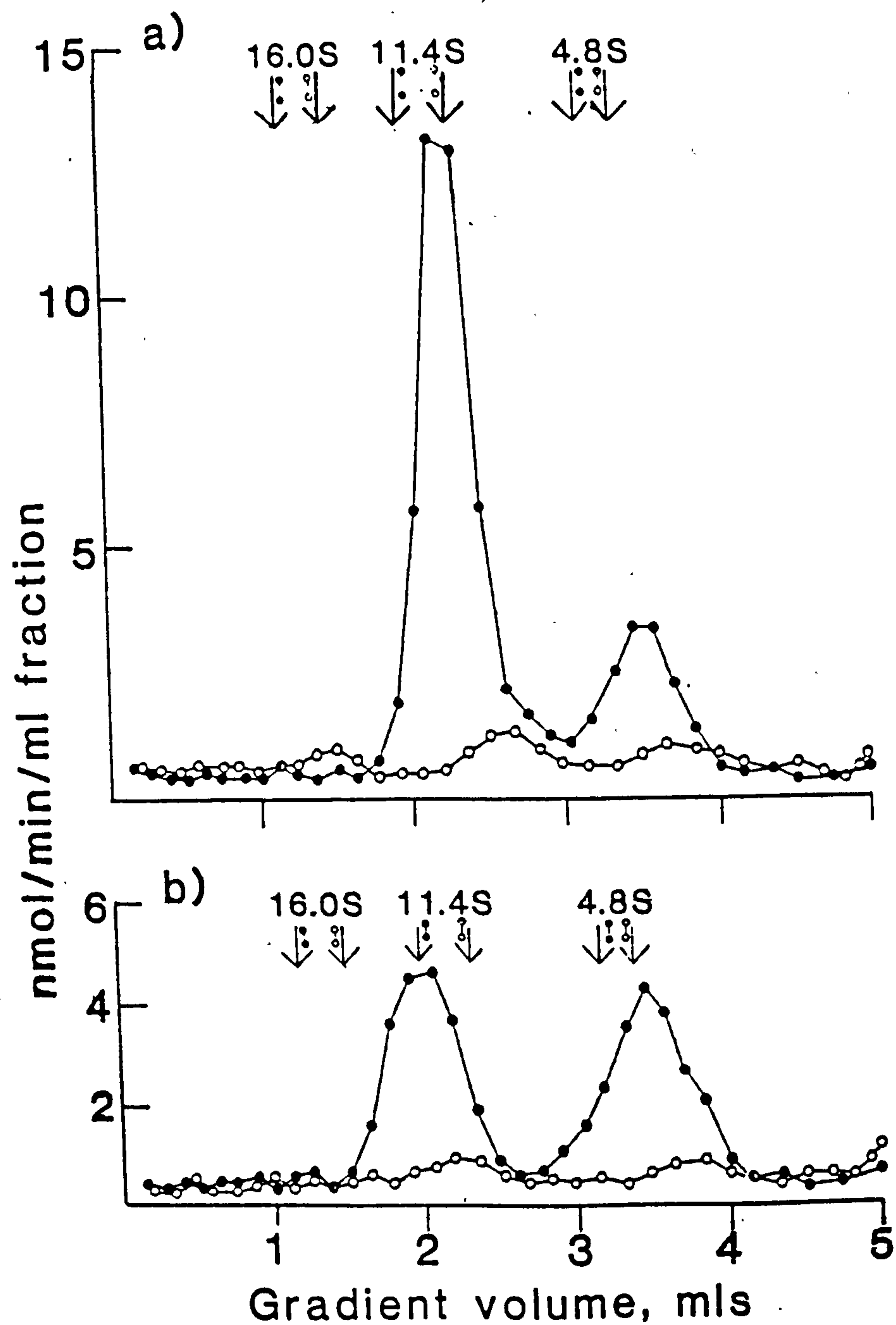


Figure 26. Extraction of globular and asymmetric molecular forms of a) AChE and b) BChE in normal elderly parietal cortex. Initial extraction in low-salt medium (●—●) solubilizes globular forms whilst re-extraction in high salt medium (○—○) solubilizes asymmetric forms. Extract loaded = 400 $\mu$ l on each gradient (derived from the equivalent of 40mg wet weight of tissue). Sedimentation velocity markers = alcohol dehydrogenase (4.8S), catalase (11.4S) and  $\beta$ -galactosidase (16.0S).

et al, 1981), these forms also appear to be globular. In addition, although both these forms were also present in the high salt extract, these probably represent globular rather than asymmetric forms (see above). Globular forms of BChE with sedimentation velocities of 11.5S and 3.5S correspond to  $G_4$  and  $G_1$  quaternary structures respectively. It therefore appears that the assumption made earlier (section II.3.f) is valid and that although the  $A_4$  and  $G_4$  molecular forms of AChE and BChE have similar sedimentation velocities, the 10S AChE and 11S BChE enzyme represent globular rather than asymmetric tetrameric forms of the enzymes.

#### ii) Extractions in the absence and presence of detergent

The extractions in the absence and presence of detergent (figure 27) were carried out on samples of parietal cortex taken from two elderly normal cases. Since the results obtained were essentially the same, results from only one of these cases are shown in figure 27. The results clearly demonstrate that the majority (89%,  $n=2$ ) of the  $G_4$  form of AChE and only about half (55%,  $n=2$ ) of the  $G_1$  form required detergent for their extraction and are therefore presumably membrane bound. In contrast to the  $G_4$  form of AChE, the proportions of the  $G_4$  form of BChE that were soluble and membrane-bound were approximately equal whilst the  $G_1$  form of BChE exists in approximately equal quantities of soluble and membrane-bound forms and is therefore similar to the corresponding form of AChE.

### 3. Molecular forms of cholinesterases in the pathological central nervous system: Comparison with the normal

#### a) Molecular forms of acetyl- and butyryl-cholinesterase in normal and SDAT parietal cortex.

The activities of both AChE and BChE at various stages of the extraction and analyses of the molecular forms are presented in table 19 which shows that in the present series of cases, the total levels of AChE activity in the crude homogenates of SDAT cortex are significantly reduced by 45% ( $P<0.001$ ) compared to the normal. Furthermore solubilization of the AChE activity present in the crude homogenates was greater than 90% in both groups and the amount of activity recovered from the gradients (75-80%) did not differ significantly between the groups.



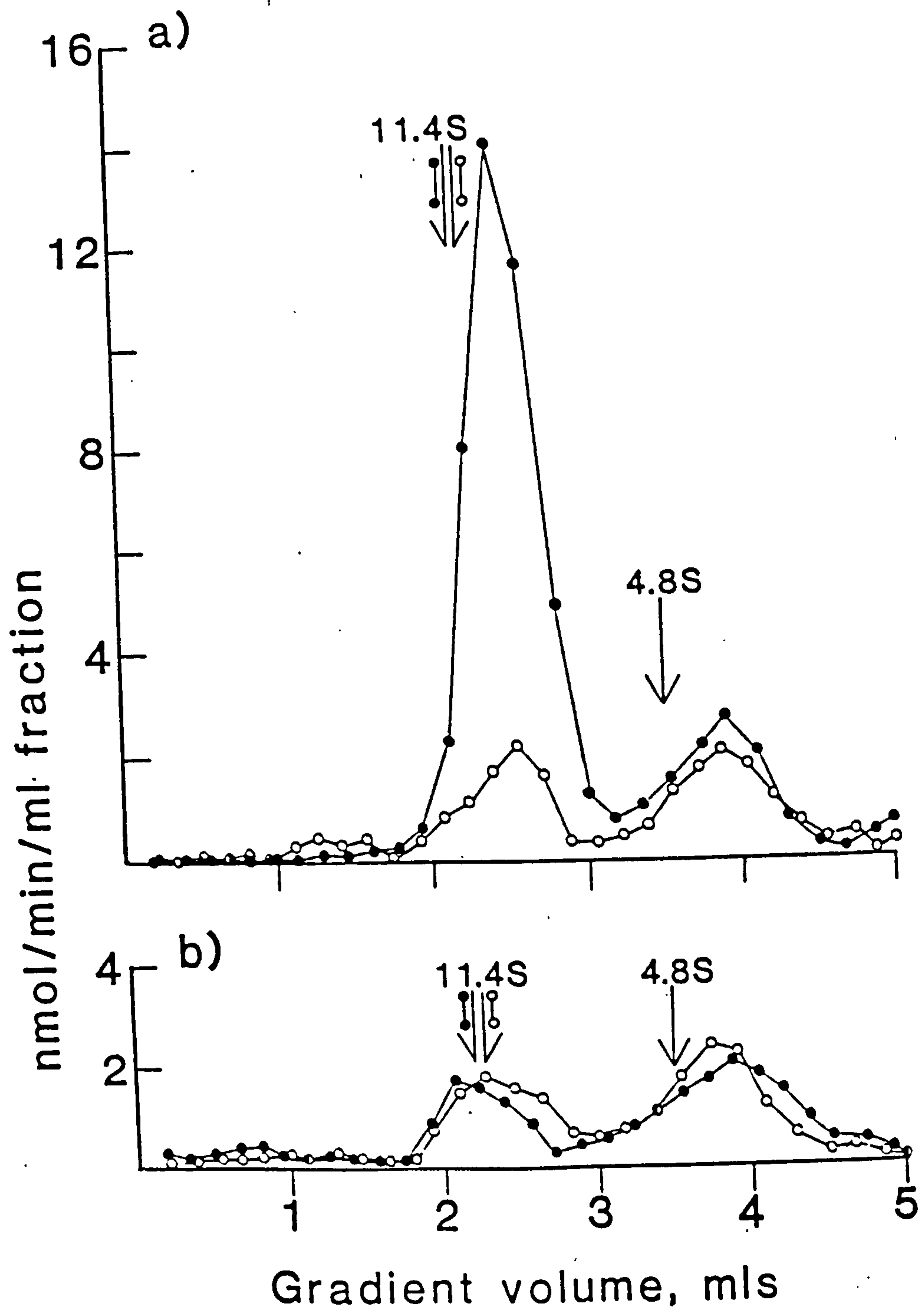


Figure 27. Extraction of soluble and membrane bound molecular forms of AChE (upper figure) and BChE (lower figure) in normal elderly parietal cortex. Initial extraction in absence of detergent (O—O) represents soluble forms whilst re-extraction in detergent-containing medium (●—●) solubilized hydrophobic membrane-bound forms. Extract loaded = 400 $\mu$ l on each gradient (derived from the equivalent of 40mg wet weight of tissue). Sedimentation velocity markers = alcohol dehydrogenase (4.8S), catalase (11.4S) and  $\beta$ -galactosidase (16.0S).

Table 19

Levels of activities, percentage solubilization and recovery of activities of both acetyl- and butyryl-cholinesterase in normal and Alzheimer-type parietal cortex<sup>a</sup>

ACETYLCHOLINESTERASE						
	Enzyme solubilization			Recovery from gradient		
	Homogenate nmol/min/ml	Supernatant nmol/min/ml	%solub- ilized	<sup>b</sup> Loaded mU	<sup>b</sup> Recovered (%) mU	
Normal	51.3 ± 5.2	47.6 ± 7.3	92.8	19.0	15.1	(79)
SDAT	*** 28.3 ± 7.8	25.5 ± 7.6	90.1	10.2	7.7	(75)

BUTYRYLCHOLINESTERASE						
	Enzyme solubilization			Recovery from gradient		
	Homogenate nmol/min/ml	Supernatant nmol/min/ml	%solub- ilized	<sup>b</sup> Loaded mU	<sup>b</sup> Recovered (%) mU	
Normal	24.7 ± 6.2	24.2 ± 7.9	98	10.4	7.0	(67)
SDAT	30.1 ± 7.4	27.8 ± 8.5	92	11.1	7.7	(79)

<sup>a</sup> Values shown = mean ± standard deviation (n = 5, both groups)

<sup>b</sup> mU = nmol of substrate hydrolysed/minute

\*\*\* Significantly different (p<0.001) from the normal (Student's t-test with F-test of variances).



In contrast to AChE, the total BChE levels in the parietal cortex homogenates did not differ significantly between normal and SDAT cortex. The solubilization of BChE was not significantly different between the normal (98%) and SDAT (92%) cortex. In addition, the BChE activity recovered from the gradients was also similar (approximately 70%) for both groups.

Figures 28 and 29 illustrate the sedimentation profiles of AChE and BChE obtained by analysis of the five normal and five SDAT cases and an analysis of the data is presented in table 20. There was no tendency in the normal cortex for either the levels or distribution of molecular forms of AChE or BChE to be associated with patient age, sex or postmortem delay. The patterns of distribution of AChE in the normal cortex in the different individuals are very similar with the major form - comprising on average 66.6% (range 61-72%) of the activity recovered - sedimenting with an S value of 9.8S ( $G_4$ ). The remaining activity consisted of a slower sedimenting form with an S value of 3.5S ( $G_1$ ) and totalling 31.5% of the recovered activity and a high molecular weight ( $A_{12}$ ) form, representing 1.9% of the total, sedimented within 250 $\mu$ l of the 16.0S  $\beta$ -galactosidase marker.

In the SDAT cortex, all three forms were detected with sedimentation velocities very similar to those seen in the normal cortex. Analysis of the activities of the individual peaks shows that whilst the activity of the  $G_1$  and  $A_{12}$  forms did not differ from those found in the normal, the  $G_4$  form was significantly ( $P < 0.001$ ) reduced to levels of only 27% those found in the normal. Interestingly, one of the SDAT cases (H.M.) showed the presence of a form of AChE that had a sedimentation velocity (6S) between that observed for the  $G_1$  and  $G_4$  forms and was not seen in any of the normal brains yet constituted 50% of the total AChE recovered from this particular case. Furthermore, neither the age (78 years), postmortem delay (31 hours) nor mean plaque count (41.3) distinguished this patient from any of the other SDAT subjects. This patient did, however, have a short (less than one year history) of the disease.

Unlike AChE, BChE was found to be present only as two forms in the normal parietal cortex; one form sedimenting slightly faster than the  $G_4$  form of AChE (11.5S as opposed to 10S for AChE) and a lower molecular weight form sedimenting at a similar velocity (3.6S) to the  $G_1$  form of AChE. Furthermore, unlike the distribution of activity between the corresponding AChE forms, the  $G_1$  and  $G_4$  forms of BChE were present in approximately equal amounts. In the SDAT cortex, the same two forms were

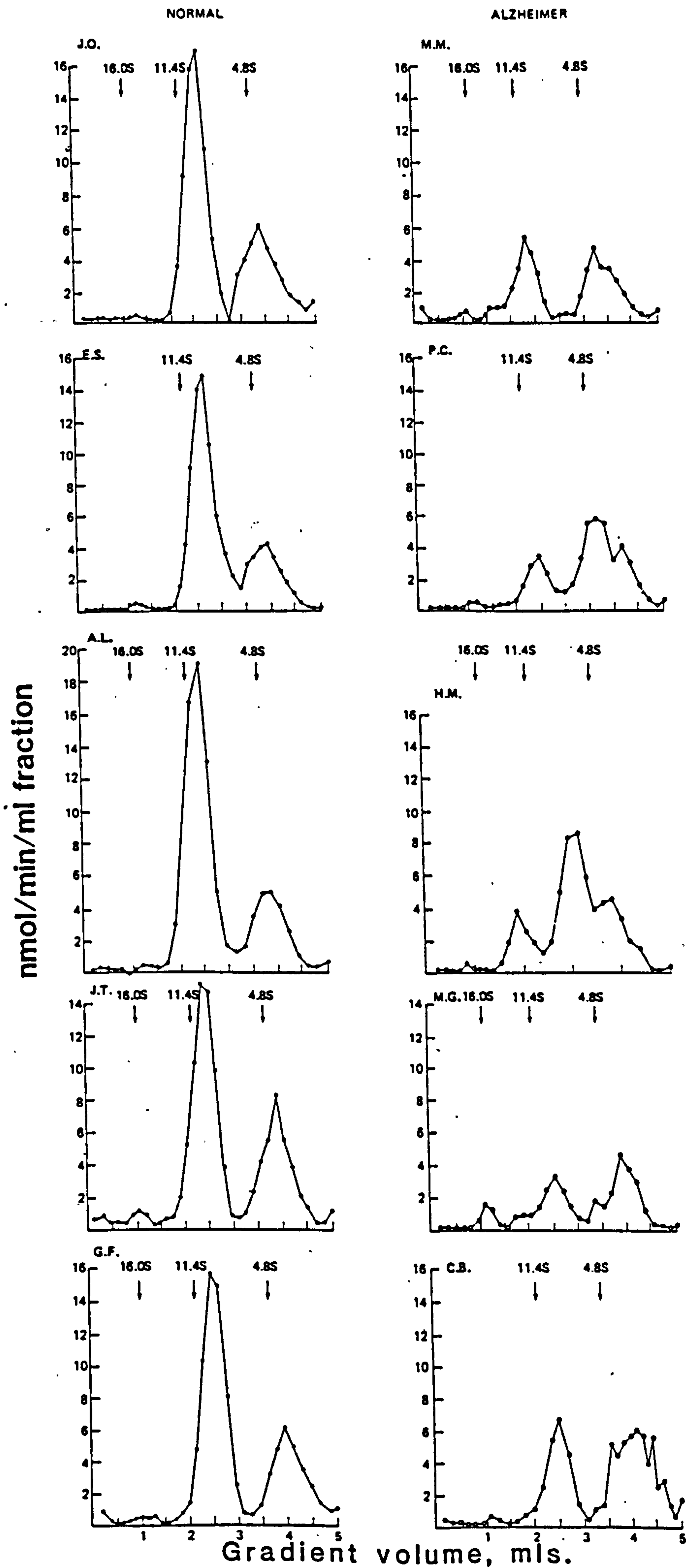


Figure 28. Sedimentation profiles of the molecular forms of AChE in parietal cortex of five normal (left) and five SDAT (right) cases. Extract loaded = 400 $\mu$ l in each case (derived from the equivalent of 40mg wet weight tissue). Sedimentation velocity markers = alcohol dehydrogenase (4.8S), catalase (11.4S) and  $\beta$ -galactosidase (16.0S).



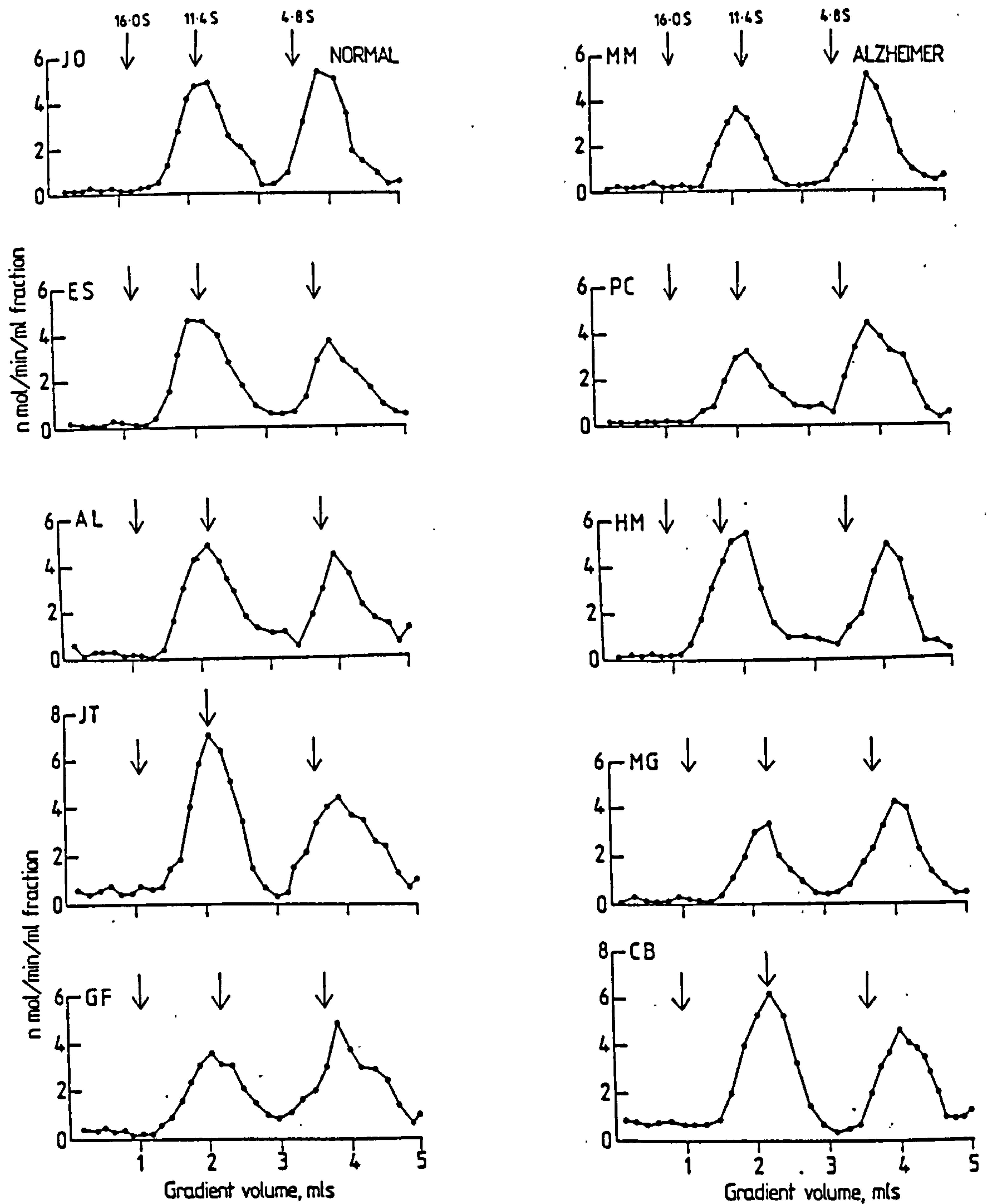


Figure 29. Sedimentation profiles of the molecular forms of BChE in parietal cortex of five normal (left) and five SDAT (right) cases. Extract loaded = 400 $\mu$ l in each case (derived from the equivalent of 40mg wet weight of tissue). Sedimentation velocity markers = alcohol dehydrogenase (4.8S), catalase (11.4S) and  $\beta$ -galactosidase (16.0S).

Table 20  
Distribution and sedimentation coefficients of acetylcholinesterase and butyrylcholinesterase molecular forms in normal and SDAT parietal cortex <sup>a</sup>

ACETYLCHOLINESTERASE						
	G <sub>1</sub> form		G <sub>4</sub> form		A <sub>12</sub> form <sup>a</sup>	
	<sup>b</sup> S value	<sup>c</sup> Activity	S value	Activity	S value	Activity
Normal	3.48 ± 0.38	4.77 ± 0.92	9.84 ± 0.36	10.04 ± 0.98	<sup>d</sup> 16	0.29 ± 0.14
SDAT	3.44 ± 0.82	4.76 ± 1.44	10.18 ± 0.57	*** 2.68 ± 0.81	<sup>d</sup> 16	0.27 ± 0.20
BUTYRYLCHOLINESTERASE						
	G <sub>1</sub> form		G <sub>4</sub> form		A <sub>12</sub> form	
	<sup>b</sup> S value	<sup>c</sup> Activity	S value	Activity	S value	Activity
Normal	3.62 ± 0.40	3.79 ± 0.88	11.50 ± 0.42	3.96 ± 1.06	Not detected	
SDAT	3.34 ± 0.26	3.60 ± 0.66	11.46 ± 0.80	3.42 ± 1.27	Not detected	

<sup>a</sup> Values shown are mean ± standard deviation (n = 5, both groups)

<sup>b</sup> S value = sedimentation velocity (Svedberg units)

<sup>c</sup> Units of activity = nmol hydrolysed/minute

<sup>d</sup> The S value of this particular form could not be reliably estimated since the gradient was not linear in the region where this form was recovered (section III.1.a). It did, however, sediment in close proximity to the 16.0S β-galactosidase marker enzyme

\*\*\* Significantly different (P<0.001) from the normal (Student's t-test with F-test of variances).



also found to be present and were similar to the normal in respect to both their relative amounts and absolute activities.

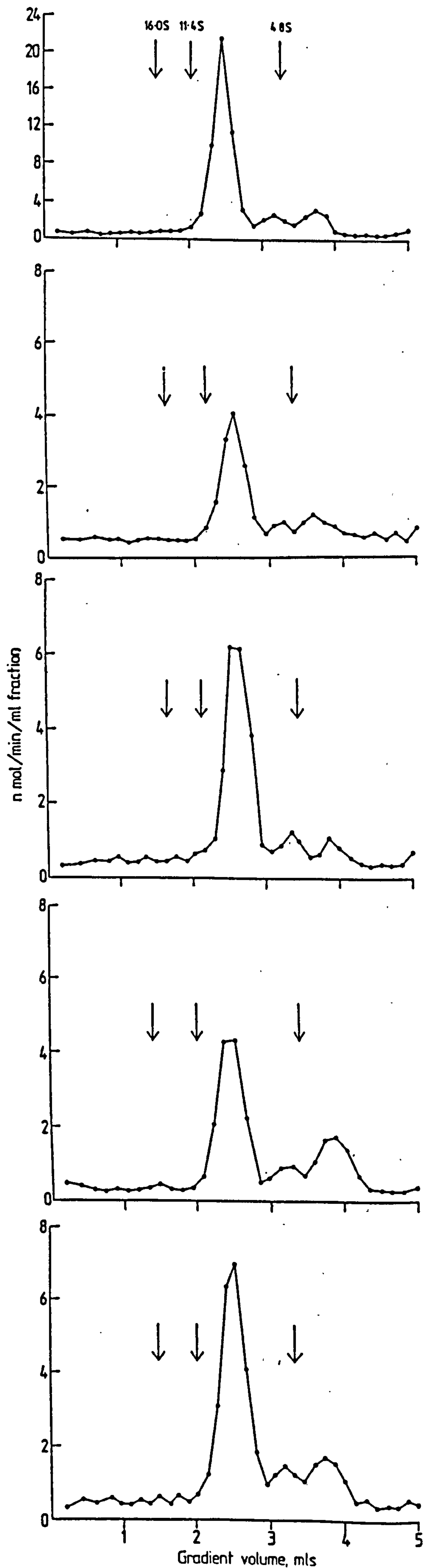
b) Molecular forms of AChE in normal and SDAT nucleus of Meynert.

Since there was a striking loss of the  $G_4$  form of AChE from the parietal cortex, the molecular forms of AChE were further examined in the subcortical nucleus of Meynert in SDAT. The mean activities of the normal and SDAT homogenates ( $\pm$  standard deviation) were  $188 \pm 123$  and  $70.7 \pm 9.9$  nmol/mg protein respectively. Whilst there is a loss of AChE activity in the SDAT group of 62%, it should be pointed out that the three SDAT cases had levels of activity similar to four of the five normal cases and that the mean activity of the normal group is biased by a single case with relatively high activity. The solubilization of AChE from the homogenate into the 20,000g supernatant was, where measured, greater than 80% with no difference between normal and SDAT cases. The recovery of activities from the gradients was greater than 85% and again there was no difference between the normal and SDAT groups. The normal distribution of AChE in the nucleus of Meynert (figure 30 and table 21) shows that the major portion of the activity (77.5%) had a sedimentation velocity of 9.1S ( $G_4$  form), whilst the remainder of the activity resolved as two lower molecular weight forms with sedimentation velocities of 5.3S ( $G_2$ ) and 3.5S ( $G_1$ ) which accounted for 8.1% and 14.4% of the total activity respectively. In addition, there was no tendency for neither the total activity nor the distribution of molecular forms to be related to either patient age, sex or postmortem delay.

In SDAT, the loss of activity in the homogenates of 62% is reflected by a loss of activity recovered from the gradients of 56%. The same three forms present in the normal cases are also present in the SDAT tissue with very similar sedimentation velocities. The striking feature of the nucleus of Meynert compared to the cortex in SDAT is that not only is an additional  $G_2$  form of AChE present but also the distribution of activity, although over 50% lower for each particular form, is virtually identical to that seen in the normal with the  $G_4$  form accounting for 76.7% of the total activity whilst the slower sedimenting  $G_2$  and  $G_1$  forms constitute 8.3% and 15.0% of the total activity respectively.

c) Molecular forms of AChE and BChE in parietal cortex of cases of Parkinson's disease with dementia

Figure 31 shows the distribution of the molecular forms of both AChE



## ALZHEIMER

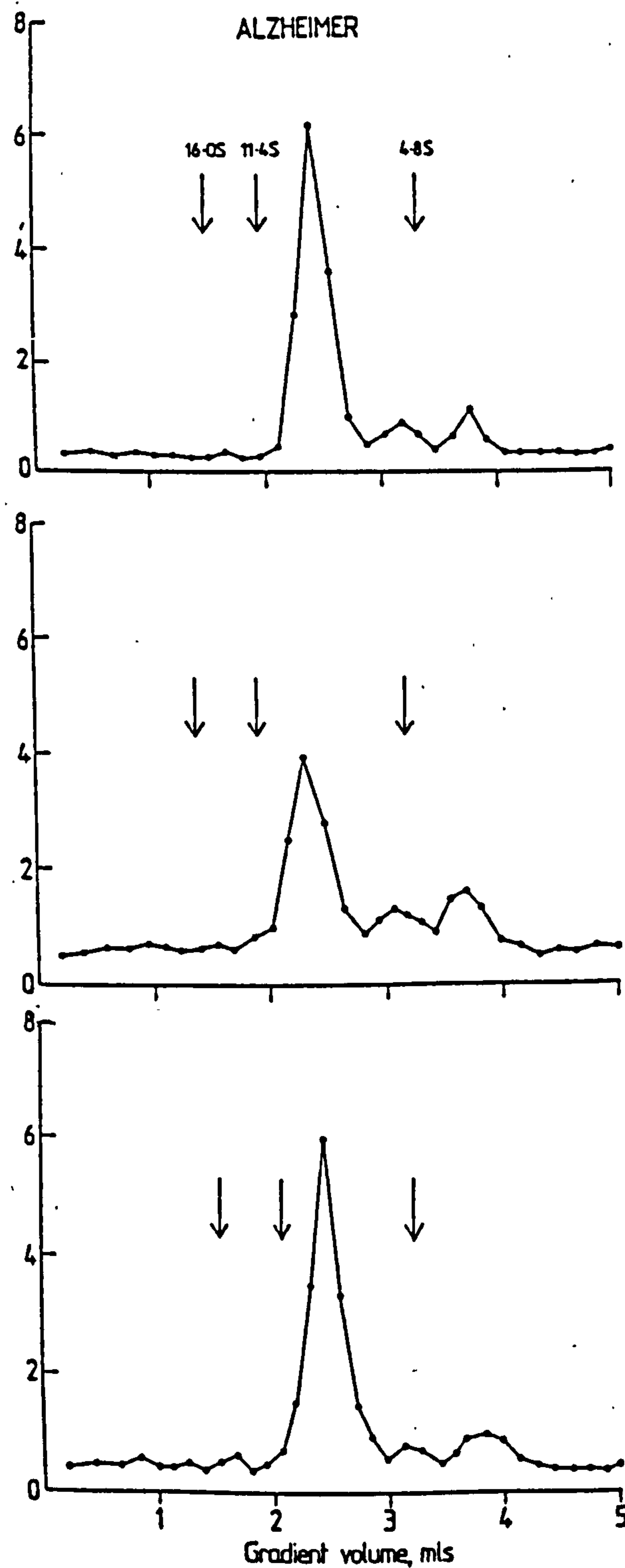


Figure 30. Distribution of molecular forms of AChE in the nucleus of Meynert of normal (left hand side) and SDAT (right hand side) cases. Volume of extract loaded onto each gradient is derived from the same amount of tissue (equivalent to 28 $\mu$ g protein) in each case analysed. Sedimentation velocity markers = alcohol dehydrogenase (4.8S), catalase (11.4S) and  $\beta$ -galactosidase (16.0S).



Table 21  
Comparison of the molecular forms of  
acetylcholinesterase in normal and SDAT nucleus of Meynert <sup>a</sup>

Sedimentation properties						
	G <sub>1</sub>		G <sub>2</sub>		G <sub>4</sub>	
	<sup>b</sup> S value	<sup>c</sup> Activity	S value	Activity	S value	Activity
Normal	3.47 ± 0.31	0.59 ± 0.29	5.25 ± 0.35	0.33 ± 0.18	9.06± 0.18	3.17 ± 1.97
SDAT	3.39 ± 0.37	0.27 ± 0.14	5.12 ± 0.28	0.15 ± 0.03	3.17 ± 0.15	1.38 ± 0.23

Proportion of total activity recovered			
	G <sub>1</sub>	G <sub>2</sub>	G <sub>4</sub>
Normal	14.4%	8.1%	77.5%
SDAT	15.0%	8.3%	76.7%

<sup>a</sup> Values shown are mean ± standard deviation (normal group, n = 5; SDAT group, n = 3)

<sup>b</sup> S value = sedimentation velocity (Svedberg units)

<sup>c</sup> Activity = nmol hydroysed/min

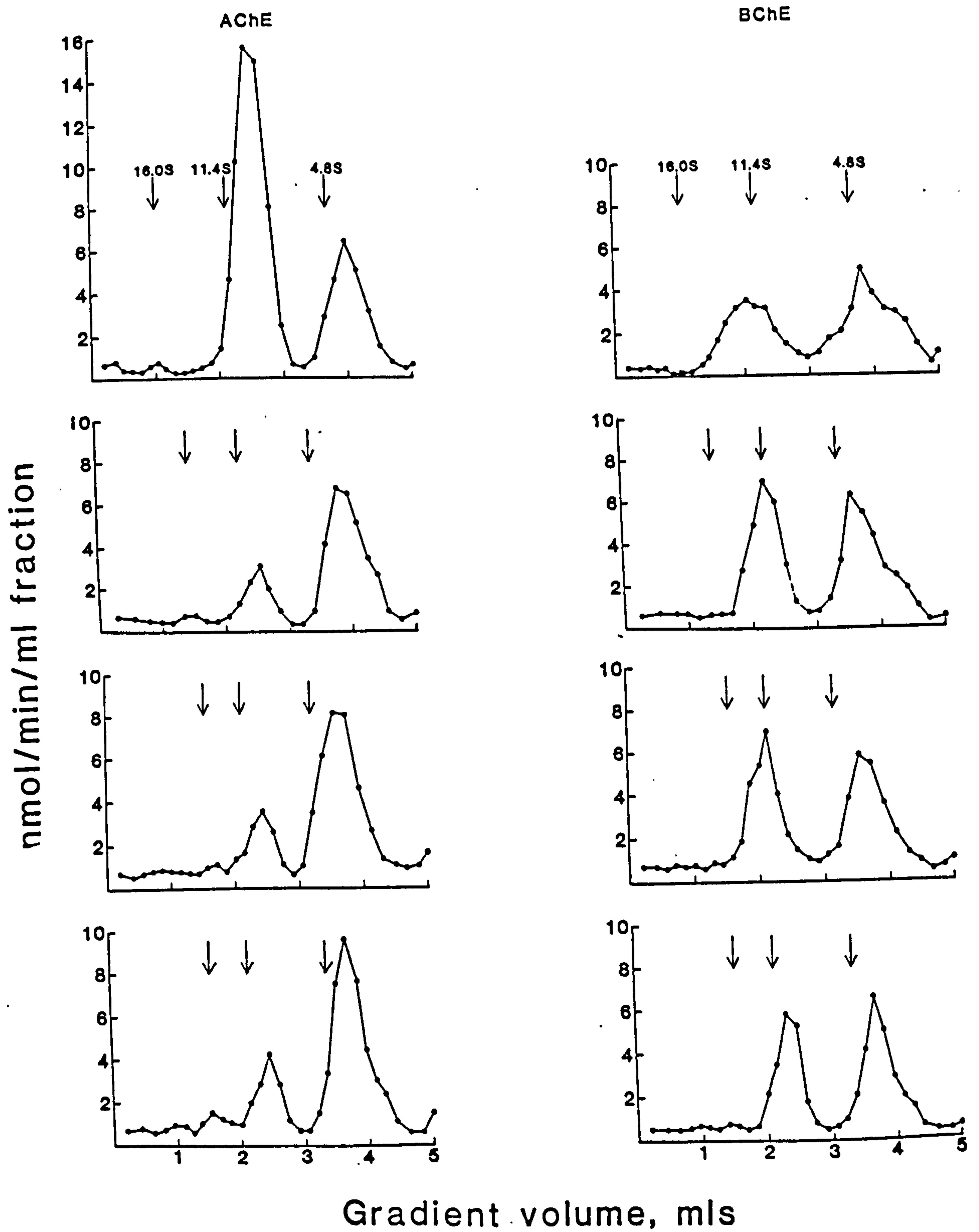


Figure 31. Sedimentation profiles of molecular forms of AChE (left hand side) and BChE (right hand side) in one normal case (top) and three Parkinsonian patients with dementia (lower diagrams). Load = 400 $\mu$ l of extract (derived from the equivalent of 40mg wet weight of tissue). Sedimentation velocity markers = alcohol dehydrogenase (4.8S), catalase (11.4S) and  $\beta$ -galactosidase (16.0S).



Table 22  
Comparison of molecular forms of acetyl- and butyryl-cholinesterase in  
postmortem parietal cortex from normal subjects and demented Parkinsonian cases<sup>a</sup>

ACETYLCHOLINESTERASE						
	G <sub>1</sub> form		G <sub>4</sub> form		A <sub>12</sub> form	
	<sup>b</sup> S value	<sup>c</sup> Activity	S value	Activity	S value	Activity
Normal <sup>d</sup>	3.48 ± 0.38	4.77 ± 0.92	9.84 ± 0.36	10.04 ± 0.98	<sup>e</sup> 16	0.29 ± 0.14
Parkinson's disease	3.67 ± 0.13	5.00 ± 2.00	9.63 ± 0.11	5.00 ± 2.00	<sup>d</sup> 16	0.20 ± 0.10
BUTYRYLCHOLINESTERASE						
	G <sub>1</sub> form		G <sub>4</sub> form		A <sub>12</sub> form	
	<sup>b</sup> S value	<sup>c</sup> Activity	S value	Activity	S value	Activity
Normal <sup>d</sup>	3.62 ± 0.40	3.79 ± 0.88	11.50 ± 0.42	3.96 ± 1.06	Not detected	
Parkinson's disease	3.83 ± 0.12	5.00 ± 2.00	10.98 ± 0.72	5.00 ± 2.00	Not detected	

<sup>a</sup> Values show are mean ± standard deviation (normal group, n = 5; Parkinsonian group, n = 3)

<sup>b</sup> S value = sedimentation velocity (Svedberg units)

<sup>c</sup> Units of activity = nmol hydrolysed/min

<sup>d</sup> Normal data are derived from table 20

<sup>e</sup> The S value of this particular form could not be reliably estimated since the gradient was not linear in the region where this form was recovered (section III.1.a). It did, however, sediment in close proximity to the 16.0S β-galactosidase marker enzyme.

BChE in the parietal cortex of three cases of Parkinson's disease accompanied by dementia, compared to a typical normal distribution of both AChE and BChE (see figures 28 and 29 for distribution of additional normals). An analysis of the data is presented in table 22 (normal data derived from table 20) and it should be noted that the two groups are not age-matched (table 14).

The percentage of enzyme solubilized (greater than 90%) and the recovery of activity from the gradient (73% for AChE, 68% for BChE) were similar to those observed for normal and SDAT parietal cortex. Three forms of AChE were detected in the demented Parkinsonian cortex with similar sedimentation velocities to the three forms observed in normal cortex. The loss of activity in the Parkinsonian cortex (mean homogenate activity = 225 nmol/min/g wet weight versus normal activity of 513 nmol/min/g wet weight) was due, as was the case for the SDAT cortex, to an extensive (85%) and selective loss of the  $G_4$  form of AChE whilst the levels of activity of the heavy and light forms remained essentially unaltered.

The BChE activity in the demented Parkinsonian cortex was observed to be distributed approximately equally between the  $G_4$  and  $G_1$  species with sedimentation velocities similar to those observed in both the normal and SDAT cases. Similar to the situation in SDAT, the levels and distribution of activity of these two forms did not differ significantly between the normal and pathological situation.

#### d) Molecular forms of AChE in fornix-lesioned rats

Figure 32 illustrates the proportions of AChE in the hippocampus of a sham operated animal that are soluble and membrane bound. The situation is similar to that observed in the human parietal cortex with the majority (92%) of the  $G_4$  form and 40% of the  $G_1$  form being membrane bound.

Figure 33 illustrates the distribution of molecular forms of AChE in the hippocampus of sham-operated, unoperated and lesioned rats. Since the unoperated and sham operated animals gave very similar results, the data from these groups were pooled and are presented in table 23 as a single control group.

Two forms of AChE,  $G_1$  and  $G_4$ , were separated in both the unlesioned and lesioned groups with S values (approximately 3.2S and 9.2S respectively) that did not differ between the two groups. In the unlesioned animals the predominant form of AChE found in the hippocampus



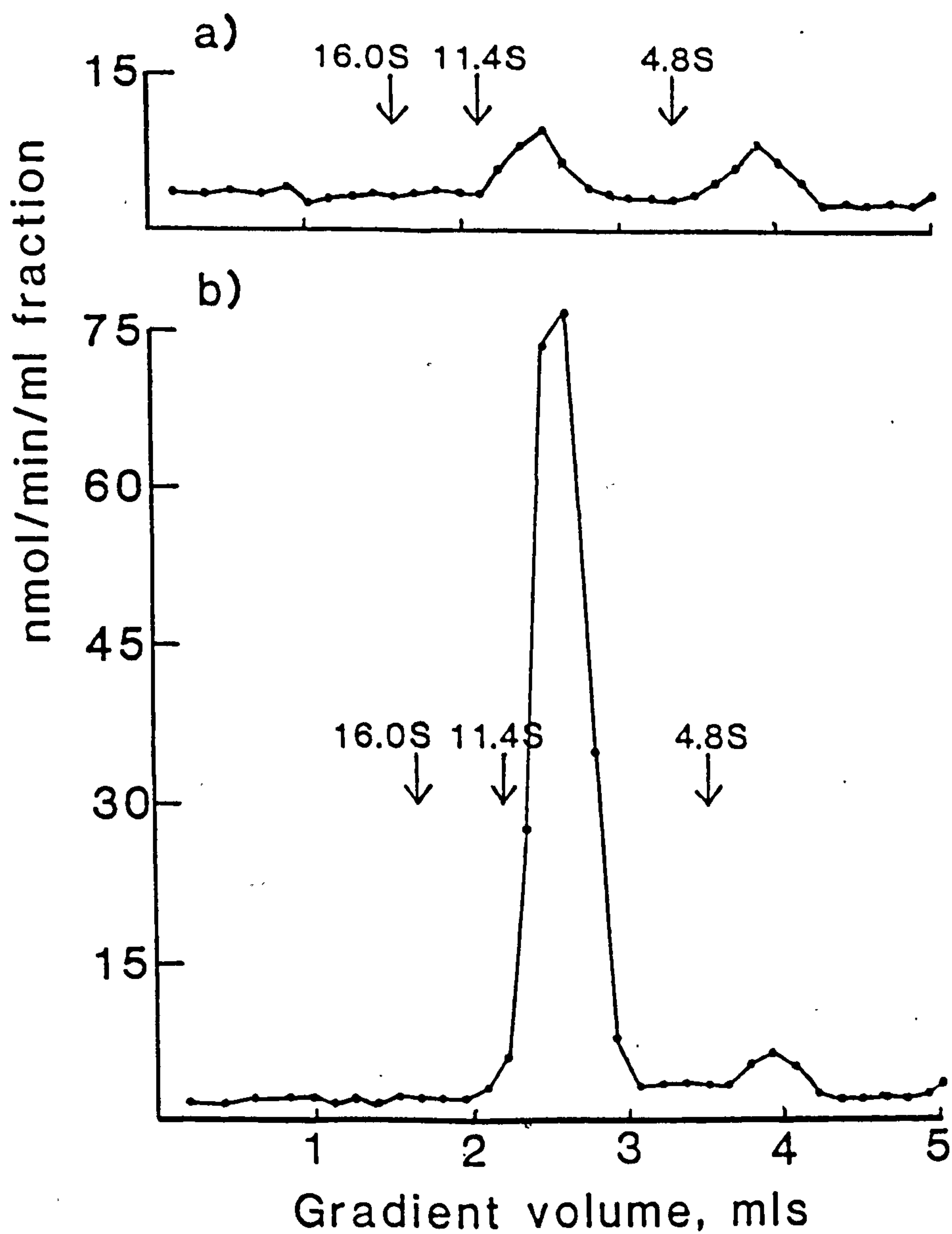


Figure 32. Diagram showing the solubility characteristics of the molecular forms of rat hippocampus AChE. a) illustrates the molecular forms of AChE extracted in the absence of detergent (= soluble AChE); b) shows the forms of AChE that were not solubilized in the initial detergent-free medium but were subsequently extracted in a detergent-containing buffer (= membrane bound AChE). Load = 150 $\mu$ l of extract derived from the equivalent of 7.5mg wet weight tissue. Sedimentation velocity markers = alcohol dehydrogenase (4.8S), catalase (11.4S) and  $\beta$ -galactosidase (16.0S).

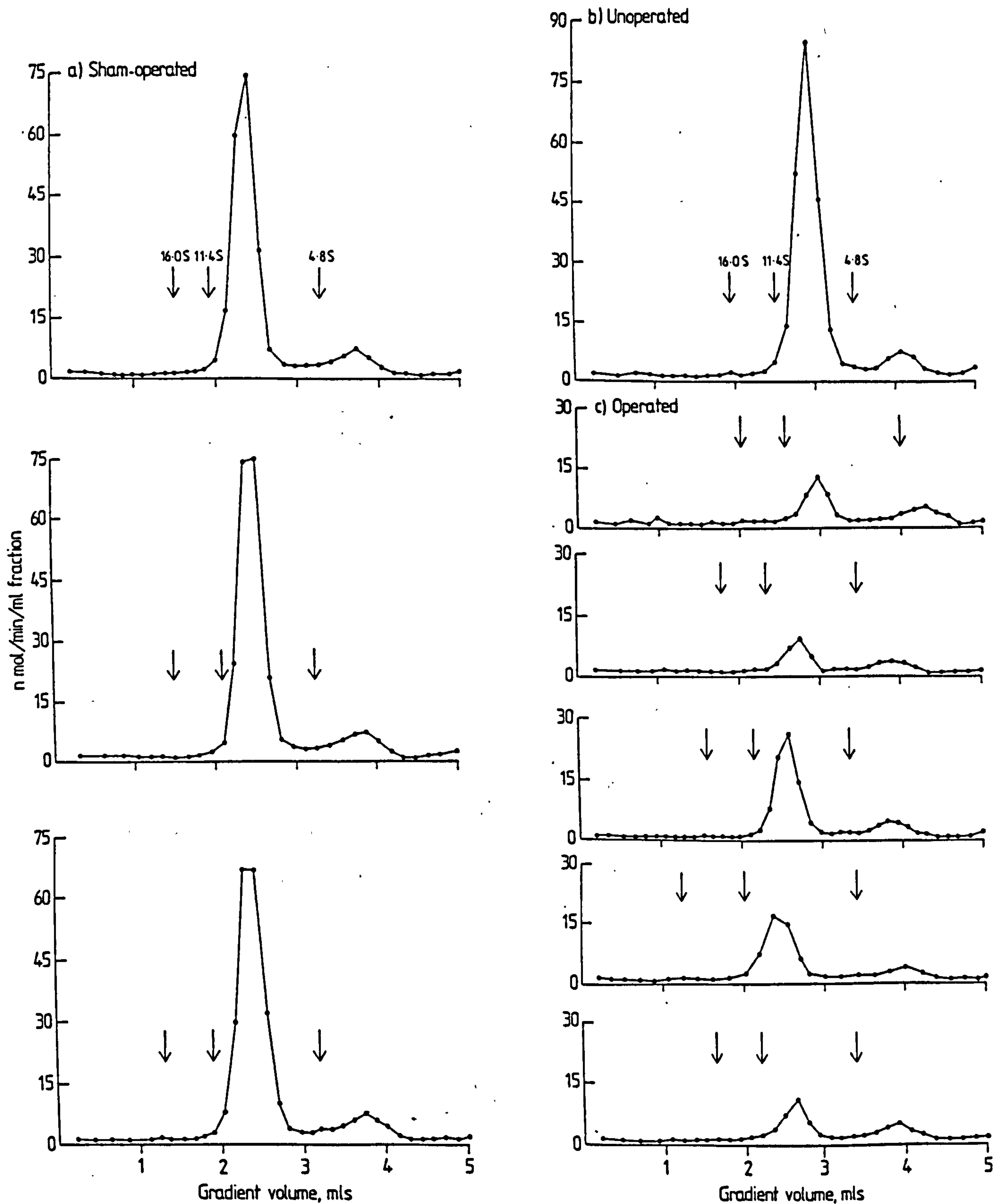


Figure 33. Diagram showing the distribution of molecular forms of AChE in the hippocampus of rats with and without surgical lesions in the septo-hippocampal pathway. a) sham operated animals; b) unoperated animal and c) operated animals. Load = 150 $\mu$ l of extract derived from the equivalent of 7.5mg wet weight tissue. Sedimentation velocity markers = alcohol dehydrogenase (4.8S), catalase (11.4S) and  $\beta$ -galactosidase (16.0S).



Table 23  
Analysis of molecular forms of acetylcholinesterase  
in the hippocampus of control and fornix-lesioned rats <sup>a</sup>

	G1 form		G4 form	
	<sup>b</sup> S value	Activity of peak, nmol/min	S value	Activity of peak, nmol/min
Control	3.26 ± 0.25	2.23 ± 0.32	9.03 ± 0.24	22.36 ± 1.20
Lesioned	3.27 ± 0.14	*** 1.27 ± 0.23	9.27 ± 0.29	*** 3.79 ± 2.35

<sup>a</sup> Values shown are mean ± standard deviation (n = 4, control group; n = 5, lesioned group)

<sup>b</sup> S value = sedimentation velocity (Svedberg units)

\*\*\* Significantly different (p<0.001) from the normal (Student's t-test with F-test of variances)

was the  $G_4$  enzyme which constituted 91% of the total activity recovered from the gradient whilst the  $G_1$  form accounted for the remaining 9%. The solubilization of AChE activity present in the homogenate was greater than 90% in both lesioned and control groups of rats. Furthermore, the recovery of activity loaded onto the gradient (approximately 75%) did not differ between the groups and the loss of activity in the hippocampal homogenates of lesioned animals of 74% (range 55-85%) is reflected by a difference of activity recovered from the gradients of 79% compared to that recovered from the gradients of the control animals. Since the  $G_4$  form constitutes the majority of the activity present in the unlesioned animal, it is not surprising to find that the activity of this form is significantly ( $p < 0.001$ ) and extensively (83%) reduced in the lesioned animals. Loss of this form is not, however, solely responsible for the loss of AChE activity since there is also a significant ( $P < 0.001$ ) although less extensive (45%) loss of the  $G_1$  form in the lesioned animals. To confirm that the loss of AChE activity did indeed reflect loss of cholinergic activity, one homogenate of the hippocampus of a lesioned animal was also assayed for ChAT and compared to the activity in homogenates from two unlesioned animals. Compared to the two unlesioned animals, the lesioned animal showed a loss of AChE activity of 63% and a loss of ChAT activity of 74% which indicates that loss of AChE in the rat hippocampus does indeed reflect a loss of cholinergic activity.



#### IV. DISCUSSION

The results described in the present chapter will be discussed in three main sub-sections based upon: 1, the results of the preliminary experiments carried out to establish the suitability of postmortem human brain to studies of the molecular forms of AChE and BChE; 2, the distribution of molecular forms in the normal CNS and 3, the changes in the distribution of molecular forms in the pathological CNS.

##### 1. Preliminary experiments to determine suitability of human postmortem brain for density gradient analysis

###### a) Postmortem stability of molecular forms of AChE and BChE

The analyses of freshly excised mouse brains demonstrated the occurrence of a major  $G_4$  form of AChE accompanied by a less abundant  $G_1$  form in proportions very similar to those previously reported for mice (Rieger et al, 1976; Rieger et al, 1980). The analysis of BChE showed that  $G_4$  and  $G_1$  forms of this enzyme were approximately equally abundant. However, in a previous report "no appreciable amounts of activity" were detected (Rieger et al, 1976a) and whilst their extraction and centrifugation methods are similar to those used in the present report, it is not possible to compare the different BChE assay procedures, which are a likely cause of this discrepancy, since Rieger and colleagues did not describe their assay technique in detail. In the mouse brains which were analysed after a period of 31 hours postmortem rather than immediately after death, the AChE and BChE forms remained essentially the same, with distributions and levels of activity similar to those observed immediately after death. It would therefore appear that not only are total levels of activity stable postmortem but also no significant alterations in the distributions of molecular forms of either AChE or BChE occur. Thus, these data indicate that proteolysis and interconversion of molecular forms of either AChE or BChE do not occur to any significant extent in the intact tissue. Furthermore, the observed stability of the molecular forms of both AChE and BChE in the mouse brain suggests that the human postmortem brain is suitable for such studies.



b) Effects of protease inhibitors on molecular forms of human cortical AChE and BChE

Having established that the molecular forms of both AChE and BChE are stable and do not interconvert in the intact tissue, and to determine whether or not the molecular forms interconvert due to proteolytic enzymes present in the crude homogenate and extract, a series of experiments were carried out in which the enzymes were extracted from the intact tissue in the absence and presence of protease inhibitors.

In the present study no significant effect of protease inhibitors was observed on the molecular forms of either AChE or BChE in the human neocortex. In addition, the observation that the molecular forms of both AChE and BChE are unaffected by postmortem delay suggests that endogenous proteases do not modify the molecular forms of AChE and BChE in the brains of either mice or humans. This is contrary to the observations that endogenous proteases effect the distribution of molecular forms of AChE in chicken skeletal muscles by converting the heavy forms to lighter forms (Silman et al, 1978). However, the effect of endogenous proteases may well be species dependent since the molecular forms of AChE in mouse (Rieger et al, 1983) and rat (Groswald and Dettbarn, 1983) skeletal muscle were not affected by the presence of protease inhibitors. Similarly the protease inhibitor bacitracin appears to have little effect on the molecular forms of rat brain AChE (Sung and Ruff, 1983).

Therefore, the observation that the molecular forms of AChE and BChE in the human postmortem brain are not modified by proteolytic enzymes suggests that the molecular forms observed when extractions are carried out in the absence of protease inhibitors in the high salt, detergent-containing buffer used in all the subsequent analyses are a valid reflection of the state of the enzymes in the tissue itself. Furthermore, the absence of significant changes due to proteolytic enzymes in the tissue extracts suggests that these enzymes have little effect on the molecular forms of cholinesterases in the intact tissue, which again indicates that the molecular forms of both AChE and BChE are probably stable in human postmortem brain.

c) Effects of storage on the molecular forms of AChE and BChE

The results presented here clearly show that solubilized AChE disaggregates upon storage with conversion of the  $G_4$  form into  $G_2$  and/or  $G_1$  species whilst BChE molecular forms appear to be much more stable. A similar disaggregation of heavy to light forms upon storage has been



reported to occur for AChE in rat brain (Rieger and Vigny, 1976) and superior cervical ganglia (Gisiger et al, 1978) homogenates, and for both AChE and pseudoChE in chicken muscle homogenates (Lyles et al, 1982). In contrast, aggregation rather than disaggregation of AChE has been reported to occur when extracts of both mouse (Adamson et al, 1975) and bovine (Hollunger and Niklasson, 1973; Grassi et al, 1982) brains were stored several days at 4°C rather than being frozen. Whether the aggregation or disaggregation properties of the molecular forms are dependent upon the temperature (i.e. 4°C or -20°C) or is species dependent is not clear, however the point is clearly made that solubilized AChE and BChE should be analysed immediately after extraction. Consequently, in the present study all analyses were carried out immediately after the enzymes had been extracted from the tissue.

## 2. Molecular forms of cholinesterases in the normal central nervous system

In the present section the molecular forms of cholinesterases observed in the normal human and rat central nervous system will be discussed with particular reference to regions analysed in the pathological situations (i.e. the human parietal cortex and nucleus of Meynert and the rat hippocampus).

The recovery of activity from the gradients of greater than 70% for rat brain AChE and human AChE and BChE are comparable with the recovery after centrifugation of 70-80% of rat brain AChE (Rieger and Vigny, 1976). Similarly, a recovery of 60-90% of rat nervous and muscle AChE has been reported by Fernandez et al (1979) whilst Rieger et al (1983) reported a recovery of greater than 50% of the total mouse muscle AChE loaded onto the gradient.

### a) Overall view of the molecular forms in human central nervous tissue

In order to determine the distribution of the molecular forms of AChE and BChE in the human CNS, analyses were carried out in several different regions of the human CNS including the CSF. For AChE, the activities of the homogenates of grey matter from the different brain areas and their activities relative to the caudate nucleus are similar to those previously published (table 9). Hence, greatest activity is found associated with the caudate nucleus with relatively low activities



associated with the cortical structures of the hippocampus and temporal and parietal cortex (activities 5.5, 2.6 and 1.8% of that found in the caudate nucleus respectively).

Whilst the actual levels of BChE activity in the different grey matter brain areas reported here are of the same order as those reported previously (Foldes et al, 1962; Domino et al, 1973; Cote and Krenzner, 1983), the highest levels were associated with the cerebellum rather than the caudate nucleus and therefore differ not only from the distribution of AChE activity but also from a previous study in which the cerebellar BChE activity was lower than that of the caudate nucleus (Foldes et al, 1962). Furthermore, the regional variations in activity were less than reported previously (table 9) and it may be that the older patients used in the present study along with differences in sampling procedure account for these discrepancies.

In addition, the activity of CSF AChE and BChE (approximately 12 and 5 nmol/min/ml respectively) are of the same order as those previously reported (Arendt et al, 1984; Singer et al, 1984). However, in the fornix and spinal cord the ratio of AChE to BChE is much higher than that previously reported by Cavanagh et al (1954). This discrepancy is probably due to the fact that at the substrate concentrations used by Cavanagh et al for the assay of AChE (30mM), substrate inhibition occurs and therefore their values for AChE activity are relatively low.

An analysis of the molecular forms of AChE in the different areas shows that the  $G_4$  form is the major form present in all areas and also in the CSF. The  $G_4$  form of AChE has also been reported to be the major form present in the human (Sorensen et al, 1982) and bovine (Grassi et al, 1982) caudate nucleus, various regions of the rat brain (section d) and in the whole chicken (Vigny et al, 1976; Marchand et al, 1979) and mouse (Rieger et al, 1976, 1980) brain. The remaining activity appears to be due to the  $G_1$  form (except in the parietal cortex and spinal cord where the  $A_{12}$  form is also present, and the nucleus of Meynert where the  $G_2$  form is also present).

The molecular forms of AChE and BChE in the different parts of the human CNS do not appear to be due to contamination by blood since in all regions, except the nucleus of Meynert, the normal pattern of AChE forms did not show the presence of any significant amounts of the characteristic dimeric form of AChE associated with erythrocytes (Ott et al, 1982). Furthermore, the absence of the  $G_2$  form of AChE in extracts derived from an amount of tissue over a hundred times greater (for



example, the neocortex) - in which contamination by blood is also much greater - than that used in the analysis of the molecular forms in the nucleus of Meynert make it very unlikely that the  $G_2$  form present in this area is due to contamination by erythrocyte enzyme. In addition, since plasma BChE and erythrocyte AChE are approximately equally active in human blood (chapter 4) it is reasonable to assume that, in the absence of contamination by erythrocyte AChE, plasma BChE does not contribute significantly to the observed distribution of molecular forms of BChE.

It therefore appears that the  $G_4$  form of AChE is the major form in areas of where AChE is associated with widely differing anatomical and functional cholinergic and non-cholinergic structures and is found in areas consisting of (chapter 1, section II.3); i) intrinsic cholinergic neurons (the caudate nucleus); ii) areas where the cholinergic system consists primarily of extrinsic nerve terminals (the hippocampus and the cerebral cortex); iii) an area that is the source of a cholinergic projection (the nucleus of Meynert); iv) a white matter area containing cholinergic axons but no cholinergic synapses (the fornix); v) an area containing cholinergic motor neurons which, unlike the above mentioned areas, project directly to muscle and therefore constitute a part of the peripheral nervous system (the spinal cord); vi) an area relatively rich in AChE compared to its cholinergic activity (the cerebellum); vii) an area not only relatively rich in AChE compared to its cholinergic activity but rich in a second neurotransmitter, dopamine (the substantia nigra) and viii) a region containing only soluble forms of AChE (the CSF).

Thus, the  $G_4$  form of AChE is not specific for regions containing extrinsic cholinergic nerve terminals since it is also the major form in regions containing intrinsic cholinergic terminals. Nor, indeed, is it necessarily restricted to regions containing cholinergic nerve terminals since it is also found in the white matter of the fornix. The  $G_4$  form is also found in areas where AChE may have a role other than that associated with cholinergic neurotransmission and where it may or may not be associated with other neurotransmitter systems. The observation that the  $G_4$  form is the main form found in both the cell bodies and nerve terminals of a central cholinergic pathway suggests that this is the main form transported down the axons. Accordingly, the  $G_4$  form of AChE was the major form detected in the fornix which carries axons of the cholinergic projection from the septum to the hippocampus.

It is interesting to note that the  $G_4$  form is the major form in the



human spinal cord, where the majority of AChE activity is associated with the motor neurons, yet is only a quantitatively minor form in extracts of the muscle itself (Carson et al, 1979). This indicates that the spinal cord motor neurons are not the major source of AChE at the neuromuscular junction. The motor neuron AChE may, however, play a trophic role in the control of neuromuscular AChE (see Massoulie and Bon, 1982) and it is therefore interesting to note that the major ( $G_4$ ) form found associated with the spinal cord is also the main form released from the rat phrenic nerve and that this release increases on nerve stimulation (Skau and Brimijoin, 1978).

The observation that the  $G_4$  form of AChE is the major form found in the human CSF agrees with previous observations on humans (Massoulie and Bon, 1982; data not shown) and assuming an analogous situation occurs in man as occurs in experimental animals (Chubb and Hodgson 1982; chapter 1, section II.8) implies that the  $G_4$  molecule is the major form of AChE secreted by the neurons of the human CNS. A comparison of the molecular forms of AChE in the human lumbar CSF and plasma (chapter 4, section III.1.c) shows that the patterns of distribution are not identical suggesting that the sources of the soluble enzyme in the plasma and CSF are not the same. Relevant to studies of AChE in the human CSF it is interesting to note that the  $G_4$  form present in the lumbar CSF may well be derived from the cholinergic neurons of the spinal cord particularly since the  $G_4$  form of AChE appears to be the main form of AChE associated with these neurons and that these neurons have been shown to secrete AChE both in vivo (Kreutzberg and Toth, 1974; Kreutzberg et al, 1975) and in vitro (Oh et al, 1977). Consequently, this may explain the limited success of measurements of AChE in CSF taken from the spinal cord as an indicator of brain AChE levels in neuropsychiatric disorders affecting the brain (Davis and Goodnick, 1983; chapter 4, sections I and IV.2.c).

In contrast to the molecular forms of AChE, the molecular forms of pseudoChE's, particularly those within the CNS, have not been extensively investigated, indeed whilst total levels of pseudoChE's have been reported in the CNS the author is not aware of any previous report concerning the molecular forms of CNS pseudoChE separated by density gradient centrifugation. As with AChE, the  $G_4$  and  $G_1$  forms of BChE were found in all areas examined. However, the ratio of  $G_4$  to  $G_1$  extracted from the various CNS areas varied less for BChE (0.65 to 4.56) than for AChE (1.66 to 21.3) indicating that, at least in the human CNS, the individual molecular forms of AChE and BChE are not regulated as closely



as initially described for chicken skeletal muscle (Silman et al, 1979). In addition, the observation that the peaks of activity were asymmetric and often possessed shoulders of activity suggests that forms other than the  $G_1$  and  $G_4$  molecules may also be present and it would be interesting to conduct further investigations to try and determine the nature of this activity.

In the CSF, as in the plasma (chapter 4, section III.1.c) the  $G_4$  form of BChE was the major form present. There was, however, a difference between the distribution of the lower molecular weight activity in the CSF and plasma that was possibly due to the large difference between the CSF and plasma with respect to the total amount of protein loaded onto the gradients. Thus, in the CSF the lower molecular weight ( $G_1$ ) form of BChE sedimented as a discrete peak whereas in the plasma, the slower sedimenting activity resolved as a broad, poorly defined band of activity that may correspond to the two forms of S value 7.4 and 5.4 described by Sketelj and Sasel (1980). Whilst these data are compatible with the plasma being a possible source of a portion of the CSF BChE activity, it is also possible that at least part of the CSF BChE derives from CNS structures since "nonspecific" ChE (pseudoChE) appears to be spontaneously released from the cat and rabbit substantia nigra and the cat (but not rabbit) caudate nucleus (Greenfield et al, 1980; Greenfield and Shaw, 1982) and it would therefore be interesting to determine the molecular forms of this spontaneously released pseudoChE activity.

In summary, an analysis of different brain areas shows that the  $G_4$  form is the predominant form of AChE found in all the areas studied and would tend to suggest that whatever the particular function of AChE in different areas of the CNS, the  $G_4$  form of AChE appears to be quantitatively and possibly functionally the most important. In contrast to AChE, the levels of activity and distribution of molecular forms of BChE vary much less from region to region and its activity is not proportional to the level of cholinergic activity suggesting that this enzyme is probably unrelated to cholinergic function.

#### b) Molecular forms of AChE and BChE in normal parietal cortex

The activities of AChE and BChE in normal human parietal cortex were 513 and 247 nmol/min/g wet weight respectively and are comparable with the activities observed by Foldes et al (1962) of 529 and 134 nmol/min/g wet weight for AChE and BChE respectively. In addition, similar levels



of activity (AChE, 833; BChE, 467, units as above) have also been reported in parietal cortex of monkeys (Rosenberg and Echlin, 1965).

In agreement with the observations made on rat brain (Rieger and Vigny, 1976) the distribution and activities of the molecular forms of AChE in the individual samples of normal human neocortex were found to be very consistent. This is in contrast to the situation in human muscle where marked variations in the distribution of AChE occurs in the same muscle of different individuals (Carson et al, 1979). The molecular forms of AChE found in the human parietal cortex AChE sedimented as three discrete peaks of activity representing  $G_1$ ,  $G_4$  and  $A_{12}$  quaternary structures. The  $G_4$  form of AChE is also the most abundant form in the cerebral cortex of rats and mice (Rieger et al, 1980; Wade and Timiras, 1980). Furthermore the solubility characteristics of the  $G_4$  form of AChE indicate that in the parietal cortex the majority (89%) of this form is membrane-bound. Similarly, the  $G_4$  form is also predominantly membrane-bound in the human (Sorensen et al, 1982) and bovine (Grassi et al, 1982) caudate nucleus and is also mainly membrane-bound in rat (section d) and chicken (Vigny et al, 1976) brains. In addition, in mouse and chicken neuronal cultures the  $G_4$  form is also associated with the plasma membranes (Lazar and Vigny, 1980; Taylor et al, 1981) where it is externally facing.

Initially, asymmetric forms of AChE could not be detected in the CNS (for example, Rieger and Vigny, 1976) and were considered to be "essentially absent" from the CNS of higher vertebrates (Massoulie et al, 1980). More recently, however, small amounts of asymmetric forms, generally less than 2% of the total AChE, have been described in rat cerebellum and whole brain (Rieger et al, 1980; Rakonczay et al, 1981b), bovine caudate nucleus (Grassi et al, 1982), various regions of the visual system of the chicken (Rieger et al, 1980; Villafruela et al, 1980; Barat et al, 1983), mouse cerebellum (Rieger et al, 1980) and rabbit, frog and pig mesencephalon, cerebellum and cerebral hemispheres (Rodriguez-Borrajo et al, 1982). It is possible that the relative paucity of asymmetric forms within the central nervous system may be due to the conversion of in vivo asymmetric forms to globular forms on extraction due to an endogenous factor. This is, however, unlikely since Rieger and Vigny (1976) observed no significant alteration in the levels of  $A_{12}$  in  $A_{12}$ -containing homogenates incubated in the presence and absence of rat brain homogenates indicating that an  $A_{12}$ -converting factor was not present. Whilst asymmetric forms have been described in the



cerebral hemispheres (Rodriguez-Borrajo et al, 1982) the analyses were not performed specifically on the cortex and as such the present report is the first description of asymmetric AChE occurring in the neocortex.

Interestingly, the presence of the  $A_{12}$  form may vary between different cortical areas since no significant amounts of this form were detected in the human temporal cortex nor in the archicortex of the hippocampus. Furthermore, although a seasonal fluctuation in the levels of  $A_{12}$  has been reported to occur in the rat superior cervical ganglion (5% of the total in June-July but only 1% of the total in December-January; Gisiger et al, 1977), this is an unlikely reason for the different  $A_{12}$  cortical distribution since the patients used in the analysis of the molecular forms in the parietal cortex, temporal cortex and the hippocampus all died at different times throughout the year. In addition, whilst Rodriguez-Borrajo et al (1982) observed less asymmetric forms in the phylogenetically younger parts of the CNS, developmental differences would seem an unlikely reason for the presence of  $A_{12}$  AChE in the parietal but not temporal cortex since both these regions are rich in "association areas" which are characteristic of the high level of development of the cerebral cortex in man (Brodal, 1969). Developmental differences in  $A_{12}$  distribution of the type observed by Rodriguez-Borrajo et al (1982) are also an unlikely reason for the different cortical  $A_{12}$  distributions observed since, because the hippocampus is phylogenetically older than the neocortex, the  $A_{12}$  form would be expected to occur in the hippocampus not parietal cortex rather than vice versa.

In contrast to the three molecular forms observed for AChE, BChE was present as two molecular forms ( $G_4$  and  $G_1$ ) although it may be possible that an asymmetric form is present analogous to the  $A_{12}$  form of AChE but at much lower levels than its AChE counterpart - see, for example, rat superior cervical ganglia (Vigny et al, 1978b) - and therefore below the limits of detection of the present assay. The  $G_4$  and  $G_1$  forms of BChE sedimented slightly faster than the analogous  $G_4$  and  $G_1$  forms of AChE which is similar to the situation that occurs in the rat where BChE forms also sediment faster than the corresponding forms of AChE (Vigny et al, 1978b; Klinar et al, 1983) although in the chicken the BChE forms sediment slower than the analogous AChE forms (Lyles et al, 1979; Silman et al, 1979; Allemand et al, 1981). The human parietal cortex BChE activity is divided approximately equally between the  $G_4$  and  $G_1$  forms and, as such, differs from the distribution of AChE which is twice as abundant in the  $G_4$  relative to the  $G_1$  form.



c) Molecular forms of AChE in normal nucleus of Meynert

Despite the small amounts of tissue analysed (approximately 300µg wet weight) it was nevertheless possible to detect the molecular forms of AChE in discrete punches of the nucleus of Meynert. The normal levels of activity of around 19 µmol/min/g wet weight are high, although not as high as those observed in the human caudate nucleus (Perry et al, 1984b), and are compatible with other observations (chapter 1, sections II.3.c and 4.b) suggesting that neurons in this region are cholinergic.

The normal pattern of distribution of molecular forms of AChE in the nucleus of Meynert differed in several respects from the normal parietal cortex: i) the levels of activity varied markedly from case to case and is presumably a function of the microheterogeneity of the nucleus of Meynert region (Perry et al 1984b); ii) whilst, as in the cortex, the major form of AChE appears to be the  $G_4$  enzyme, the lower molecular weight 3.5S ( $G_1$  form) is much less abundant (10.4% of the total activity) than in the parietal cortex (31.5% of the total); iii) the  $A_{12}$  form of AChE which was detected in the parietal cortex was not found in any extract of the nucleus of Meynert and iv) a third form found in the normal nucleus of Meynert with a sedimentation coefficient of approximately 5.5S (the  $G_2$  form) was not present in normal parietal cortex.

Since the molecular forms of AChE appear to be assembled from single monomeric subunits (chapter 1, section II.9), it is possible that the  $G_2$  form present in the nucleus of Meynert, but not cortex, represents an intermediate pool of molecules used in the assembly of  $G_4$  molecules. However, analysis of the molecular forms of AChE present in the caudate nucleus which is an area, like the nucleus of Meynert, containing cholinergic cell bodies where a similar assembly process presumably takes place, was unable to detect any  $G_2$  form in this region. It may be, however, that the differences between the presence or absence of the  $G_2$  form in the nucleus of Meynert and the caudate nucleus is due to the fact that the nucleus of Meynert is a small anatomical region that supplies a very large region (the cortex). Consequently, the turnover of AChE molecules in this region may be much higher than in the caudate nucleus and therefore at any one time a larger proportion of the total AChE is present as the intermediate  $G_2$  form.

It is interesting to note that whilst the  $G_1$  form has a more or less identical S value in both the nucleus of Meynert and parietal cortex, the  $G_4$  form somewhat lighter in the nucleus of Meynert (S value 9.1) compared



to the form found in the cortex (S value 9.8). It is possible that this is a result of the different amounts of protein loaded onto the gradients since the extract loaded was derived from approximately 300µg and 40mg wet weight of nucleus of Meynert and cortex tissue respectively. It was not, however possible to test the effect of tissue load on the S. value obtained since 40mg samples of nucleus of Meynert could not be obtained using the present methods, nor was it possible to detect the molecular forms of AChE in an extract of only 300µg of cortex.

d) Molecular forms of AChE in normal rat hippocampus

In the sham operated group, the levels and distribution of molecular forms of AChE did not appear to be significantly different from the unoperated hippocampus which suggests that neither the anaesthetic nor the operative procedure itself alter the enzyme forms to any significant extent. This observation is in agreement with previous studies that have reported that the levels of hippocampal AChE or ChAT do not alter following sham operative procedures in the septo-hippocampal pathway (Oderfeld-Nowak et al, 1977; Fonnum and Walaas, 1978).

The observation that the major (91%) form of AChE in the normal adult rat hippocampus is the  $G_4$  form is in agreement with previous reports (Ben-Barak and Dudai, 1980; Wade and Timiras, 1980). Moreover, the ratio of  $G_4$  to  $G_1$  observed in the present study is virtually identical to that observed by Ben-Barak and Dudai (1980). In addition to the hippocampus, the  $G_4$  form is also the major form in whole rat brain (Rieger and Vigny, 1976; Rieger et al, 1976; Rieger et al, 1980) and more specifically the rat cerebral cortex, striatum - including the caudate nucleus - midbrain and spinal cord (Vigny et al, 1976b; Wade and Timiras, 1980; Sung and Ruff, 1983). It therefore appears that the rat central nervous system is very similar to the human in that in similar regions - including the hippocampus - the predominant form of AChE is the  $G_4$  molecule.

The solubility characteristics of the hippocampal AChE indicate that the majority of the  $G_4$  enzyme is membrane-bound, which is not only in agreement with previous observations in the whole rat brain (Rieger and Vigny, 1976; Rakonczay et al, 1981a,b) but is also comparable with the proportion of normal human parietal cortex  $G_4$  AChE that is membrane bound (section c). In addition, the proportion of the  $G_1$  form that is soluble (60%) is again not only similar to the proportions of soluble AChE in whole rat brain (62%: Rieger and Vigny, 1976) and different brain regions

(52 to 42%: Sung and Ruff, 1983) but is comparable with the portion (45%) of soluble  $G_1$  in the human parietal cortex.

Therefore, the distribution of molecular forms of AChE are similar in the human and rat, and the solubility characteristics of the human parietal cortex and rat hippocampus are comparable. Thus, the septo-hippocampal lesioned rat hippocampus would seem to be a good model for comparing the changes that occur in the distribution of molecular forms of AChE following experimental central cholinergic deafferentation with those that occur in SDAT as a consequence of a degeneration of cholinergic structures.

### 3. Molecular forms of cholinesterases in the pathological central nervous system: Comparison with the normal

#### a) Molecular forms of AChE and BChE in SDAT parietal cortex

The analysis of molecular forms of AChE in SDAT parietal cortex shows that the 45% reduction in activity in the homogenates is due to an extensive (73%) and selective loss of the  $G_4$  form. Since this form is mainly membrane bound in the parietal cortex, it may well be that this form is specifically associated with the membranes of cortical cholinergic axonal processes which, as judged by AChE-histochemistry, degenerate in SDAT (Perry et al, 1980, 1983a; Candy et al, 1983). Consequently, to determine whether or not the  $G_4$  form is specifically associated with these structures, it would be interesting to study the distribution of the molecular forms of AChE in cholinergic synaptosomes prepared from normal and SDAT cortex.

The observation that the  $G_4$  form of AChE is selectively lost from the cortex of SDAT subjects may prove to be of great importance in the development of an anticholinesterase that is specific for this particular form of AChE at the synapse. Hence, a  $G_4$ -specific AChE inhibitor would combine with the  $G_4$  form at the synapse and therefore specifically enhance their cholinergic functions. Whilst no anticholinesterase is known that selectively inhibits the different forms of ChE's, this is an area that has received surprisingly little attention. Thus, despite reports that the different molecular forms are catalytically equivalent (Bon and Massoulie, 1976; Vigny et al, 1978a) no systematic study has been carried out into the possible inhibitor sensitivity of the different molecular forms.



It was noted in the laminar study of AChE in normal and SDAT cortex (chapter 2) that a significant amount of cortical AChE activity does not appear to be associated with presynaptic cholinergic structures and it may well be that the  $A_{12}$  and  $G_1$  forms may be associated with such structures. Thus, the loss of cortical cholinergic axonal processes indicated by loss of AChE-staining fibres both within the cortex and the subcortical nucleus of Meynert (Perry et al, 1980, 1983a; Candy et al, 1983), would require that the  $A_{12}$  and  $G_1$  forms actually become relatively enriched in the remaining fibres, which would seem unlikely. The  $A_{12}$  and  $G_1$  forms of AChE may be located in association with postsynaptic cholinceptive structures which as judged by their muscarinic receptor binding appear to be largely unaltered (chapter 1, section I.7.a.i). In contrast, a portion of this non-cholinergic AChE may not be associated with neurons at all but may, instead, be associated with glial cells and in this context it is interesting to note that cultured rat glial cells have been shown to contain only the  $G_1$  form of AChE (Rieger et al, 1976) although there are, however, no reports concerning AChE-staining glial cells in the human neocortex.

An additional point of interest in the analysis of molecular forms of SDAT is the presence in one case (H.M.) of the 6S ( $G_2$ ) form of AChE which represented 50% of the total AChE activity recovered from the gradient. This form is unlikely to be analogous to the artefactual form of AChE, sedimenting at 6.5 to 7.0S, which appeared (Grassi et al, 1982) upon storage of extracts of bovine caudate nucleus at 4°C for two reasons: i) the extracts used in the present study were not stored prior to analysis and ii) the artefactual forms observed by Grassi and colleagues were not observed in the presence of either Triton X-100 or high salt concentrations, both of which were used throughout the present analyses. It would therefore appear that this form is real and may well represent an intermediate stage between the normal and typical SDAT distribution of molecular forms of AChE. It is therefore interesting to note that this case had a short (less than one year) history of the disease and has previously been reported to have levels of ChAT within the normal range in both the frontal cortex and the nucleus of Meynert (Perry et al, 1982b).

The levels of BChE were not significantly different in the SDAT parietal cortex which agrees with the observations made on the levels of BChE throughout the depth of the temporal cortex (chapter 2). The normal levels of BChE observed in the parietal cortex are not strictly



comparable with the previous literature reporting elevated levels of BChE in SDAT since neither Op den Velde and Stam (1976) nor Perry et al (1978a,b) specifically studied the parietal cortex.

It is interesting to note that despite the extensive loss of the  $G_4$  form of AChE in SDAT, the  $G_4$  form of BChE remains essentially unaltered. For this reason, along with the different intracortical and CNS localization of BChE and AChE (chapter 2 and this chapter, section IV.2.a) a precursor role of BChE for AChE (Koelle et al, 1976, 1977a,b) would, at least in the human neocortex, appear highly unlikely. Furthermore, these data suggest that the joint regulation of molecular forms of AChE and pseudoChE that occurs in chicken skeletal muscle (Silman et al, 1979) does not occur in the human neocortex.

In contrast to the situation in SDAT, in which a loss of presynaptic cholinergic markers, presumably indicating a degeneration of presynaptic cholinergic nerve terminals, is accompanied by a selective loss of the  $G_4$  form of AChE, the converse situation has been reported to occur during development, when cholinergic synapses are being formed. Thus, during maturation of both the rat and chicken brain there is a preferential increase in the specific activity of the  $G_4$  form of AChE whilst the lighter form ( $G_1$  form in rat,  $G_2$  in chicken) has been reported to either remain at a steady low level (Rieger and Vigny, 1976; Marchand et al, 1977, Villafruela et al, 1981) or alter much less than the  $G_4$  form (Wade and Timiras, 1980; Ben-Barak and Dudai, 1980). In addition, differentiation of cultured mouse neuronal cells is also accompanied by a marked relative increase in the  $G_4$  forms compared to the  $G_1$  (Vimard et al, 1976; Lazar and Vigny, 1980).

#### b) Molecular forms of AChE in the nucleus of Meynert in SDAT

In the nucleus of Meynert in SDAT, although the mean activities of AChE in the homogenates were lower than in the normals, the levels of activity in the normal group were biased by a single high activity case. Thus, the three SDAT cases had levels of activity comparable to four of the five normal subjects, which is contrary to previous reports of an extensive loss of AChE activity as measured both biochemically (Candy et al, 1983) and histochemically (Candy et al, 1983; Parent et al, 1984). This discrepancy could be a result of the microheterogeneity of the nucleus of Meynert (Perry et al, 1984b) combined with the small group size used in the present study. Whatever the explanation, the striking feature is that, unlike the situation in the cortex, there is not a



selective loss of the  $G_4$  form of AChE. Furthermore, analysis of the activities of the individual peaks shows that the proportion of each of the three forms is virtually identical with that found in the normal. This would seem to indicate that loss of AChE activity from the nucleus of Meynert is not due to the specific loss of any particular form, rather all three forms are lost equally. Since the  $G_1$  and  $G_2$  forms of AChE are precursors of the  $G_4$  form (chapter 1, section II.9), and all three forms are therefore metabolically related, the equal loss of all three forms may indicate that there is a "down regulation" of AChE synthesis, similar to that suggested to occur for ChAT (Perry et al, 1982b), particularly since the majority, if not all, the AChE containing cells in this region of the monkey and rat brain also contain ChAT (Eckenstein and Sofroniew, 1983; Mesulam et al, 1983).

c) Molecular forms of AChE and BChE in the parietal cortex of Parkinson's disease patients suffering from dementia

The three demented Parkinsonian cases analysed in this present study were derived from a series of Parkinson's disease patients previously reported to have decreased levels of cortical ChAT activity (Perry et al, 1983b). A similar involvement of the cortical cholinergic system in demented Parkinsonian cases has also been reported by Ruberg et al (1982) and Dubois et al (1983). The present results show that AChE activity is also lost and that this deficit - which was, as in the SDAT subjects, not accompanied by any alteration in either the levels or distribution of the molecular forms of BChE - is due to a selective loss of the  $G_4$  form of AChE that is more extensive than that observed in SDAT. Furthermore, Alzheimer-type changes were not present and cannot therefore account for these changes. Thus, it appears that there is a similar involvement of the cortical cholinergic system whether the dementia is due to Alzheimer or Parkinsonian type pathology. Indeed in Parkinson's disease, as in SDAT, neuronal loss has been reported to occur in the nucleus of Meynert (Arendt et al, 1983, Candy et al, 1983; Whitehouse et al, 1983; Nakano and Hirano, 1984) which suggests that despite the dissimilar neuropathology of Parkinson's disease and SDAT and their different locations (essentially subcortical and cortical respectively), alterations in the cholinergic projection from the nucleus of Meynert to the cortex may be a common feature of both diseases caused by the different pathological processes. Thus, in SDAT alterations in this projection may be due primarily to a retrograde loss of projecting fibres



due to cortical pathology with secondary changes in the nucleus of Meynert, whilst in Parkinson's disease the pathological process may cause a primary loss of nucleus of Meynert neurons with a secondary loss of projecting fibres. In agreement with such a hypothesis, the loss of neurons in Parkinson's disease appears to be greater than that observed in SDAT (Candy et al, 1983).

It would be interesting to examine the cortical molecular forms in the cortex of patients suffering from alcoholic dementia since these cases have also been reported to have loss of cortical cholinergic activity (Antuono et al, 1980; Norberg et al, 1980) and loss of neurons in the nucleus of Meynert (Arendt et al, 1983). In addition further analyses of the molecular forms of AChE in the substantia nigra in Parkinson's disease would not only be of great interest in understanding the pathological processes of the disease but would also provide more fundamental information regarding the relationship (for example, see Greenfield et al, 1984) between the dopaminergic neurotransmitter system (which is severely affected in the disease) and AChE in this region.

#### d) Molecular forms of AChE in the fornix-lesioned rat hippocampus

The present observations that fourteen days after lesions of the septo-hippocampal pathway there is a 74% loss of AChE activity in the hippocampal homogenates agree well with the loss of both AChE and ChAT observed in unilateral or bilateral lesions of the fornix or septum (Lewis et al, 1967; Srebro et al, 1973; Yamamura and Snyder, 1974; Oderfeld-Nowak et al, 1977; Fonnum and Walaas, 1978; Dravid and van Deusen, 1983). When analysed using sucrose density gradient centrifugation, it was found that the reduction of AChE was due to a 81% loss of the  $G_4$  form and a 55% loss of the  $G_1$  form. Similarly, the  $G_4$  form of AChE has also been reported (Ben-Barak and Dudai, 1980) to be more reduced relative to the  $G_1$  form in the hippocampus of adult rats who had received an early septal lesion (4 days postnatal) - a situation not, therefore, directly comparable with the present experiments in which the lesion was placed in the adult, rather than immature, rat.

The solubility characteristics of the hippocampal AChE suggest that loss of the  $G_4$  form represents an extensive loss of membrane-bound AChE and agrees with the observation that there is a major loss of AChE activity associated with the membrane fraction of septal lesioned hippocampal homogenates (Yamamura and Snyder, 1974). Hence it would seem that the 81% loss of mainly membrane bound  $G_4$  AChE from the



deafferentated hippocampus is analogous to the 73% loss of the primarily membrane-bound  $G_4$  form of AChE that occurs in SDAT. However, the deafferentated rat hippocampus is not comparable with the SDAT parietal cortex with respect to the  $G_1$  form of AChE. Thus, whilst septal lesions reduced the levels of the  $G_1$  form in the rat by 55% this form was apparently unaltered in the SDAT cortex. This discrepancy may be due to the fact that in the rat hippocampus the  $G_1$  form of AChE may be associated with non-cholinergic neurons that project from the septum to the hippocampus (Fibiger, 1982; Baisden et al, 1984), and that these projections are destroyed by the lesion, whereas in the SDAT cortex such an input, if it exists, may be spared by the relatively selective pathological afferent fibre degeneration.

It would therefore appear that, with respect to the behaviour of the molecular forms of AChE, deafferentation of the rat hippocampus provides a good analogy of the situation that occurs in SDAT with a comparable degree of loss of membrane-bound  $G_4$  AChE observed in both situations. However, the two pathological situations differ with respect to the loss of the  $G_1$  form and it would therefore be interesting to determine if more selective lesioning of the septo-hippocampal pathway produces a selective loss of the  $G_4$  form. For example the use of a cholinergic-specific neurotoxin (such as the ethylcholine aziridium ion: Fischer et al, 1982) analogous to the use of 6-hydroxydopamine and 5',7'-dihydroxytryptamine in the study of the catecholaminergic and indolaminergic neurons may well produce a more selective lesion of the cholinergic system in the hippocampus and possibly a better model, with respect to the loss of AChE molecular forms, of the changes that occur in the cortex of SDAT subjects.

#### e) Concluding remarks

In summary it would appear that in both SDAT and Parkinson's disease with accompanying dementia there is a selective and extensive loss of the  $G_4$  form of AChE. An analogous loss of the  $G_4$  form of AChE was also observed in the deafferentated rat hippocampus suggesting that the cortical loss of AChE in SDAT and Parkinson's disease may indeed be due to loss of afferent input to the cortex suggested by studies of the nucleus of Meynert in these two disorders. In SDAT, the nucleus of Meynert contained less AChE activity than in the normal and this reduction in activity was due to a loss of all three forms found in this region indicating that there might be a "down regulation" of enzyme

synthesis in this region.

The data presented show that in both the normal and pathological situations, BChE does not appear to be closely associated with the cholinergic system. It would therefore be interesting to determine if this enzyme is associated with other neurotransmitter systems or has a more general function within the CNS not associated with any one particular neurotransmitter system.



Chapter 4:

MEASUREMENT OF CHOLINESTERASE ACTIVITIES IN THE BLOOD  
OF SENILE DEMENTIA OF ALZHEIMER-TYPE PATIENTS

## I. INTRODUCTION

Cholinesterases are found not only in the central nervous system (CNS) but are also present in large quantities in the blood. Therefore, in addition to their use in studies of the pathology of the central nervous system (CNS) in senile dementia of the Alzheimer-type (SDAT; see chapters 2 and 3), the measurement, in the blood, of cholinesterases might be relevant to the problem of diagnosis, since they may reflect the levels of central cholinergic activity. Therefore in the present section, levels of erythrocyte acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) were examined along with plasma AChE - which has only recently been described in humans - in patients suffering from SDAT, depression and Parkinson's disease. The results are discussed with respect to whether or not these enzymes may be useful as peripheral markers of the central cholinergic deficit in SDAT.

The problems of diagnosing SDAT have been described in more detail in a previous section (chapter 1, section I.5). In brief, although an accurate diagnosis of SDAT may be reached in the more severely demented patients, at which stage characteristic features of SDAT become manifest, problems are encountered in the diagnosis of the less severely demented patient. In such patients, the diagnosis of SDAT is based primarily on the elimination of all other possible causes of dementia. Thus, in the less severe cases of SDAT, misdiagnosis may occur due to difficulties in distinguishing dementia from depressive pseudodementia, benign senescent forgetfulness and acute confusional states (Gurland and Toner, 1983). Consequently, "the development of objective markers of Alzheimer's disease would be of great usefulness" and "ultimately an accessible biochemical marker would be of greatest specificity" (Terry and Katzman, 1983). Accordingly, a wide range of parameters have been examined in both the cerebrospinal fluid (CSF) and blood in the search for a SDAT-specific diagnostic parameter (tables 24 and 25).

Within the CSF, metabolites of neurotransmitters have been examined as possible indices of central neurotransmitter systems. Thus, decreased levels of 5-hydroxyindole acetic acid (5-HIAA) and homovanillic acid (HVA) - which are metabolites of serotonin (5-hydroxytryptamine) and dopamine respectively - were observed by Gottfries et al (1969) and more recently by Soininen et al (1981b) and Palmer et al (1984), although several other groups observed no significant difference in levels between



TABLE 24

Measurement of biochemical parameters in the cerebrospinal fluid of demented subjects

Observation	References	
<u>1. Cholinergic related parameters</u>		
a) Cholinesterase levels	<sup>a</sup> Soininen et al, 1981a Arendt et al, 1984 Johnson & Domino, 1971 <sup>d</sup> Soininen et al, 1981a <sup>d</sup> Appleyard et al, 1983 Johnson & Domino, 1971 Arendt et al, 1984 Johnson & Domino, 1971 Johns et al, 1983  Yates et al, 1980 Growdon & Logue, 1982  <sup>c</sup> Mann et al, 1981 Palmer et al, 1984 Miyata et al, 1984  *Gottfries et al, 1969a Palmer et al, 1984 Parkes et al, 1974;  *Gottfries et al, 1969a Palmer et al, 1984 Gottfries et al, 1969a Bowen et al, 1981 Wood et al, 1982  Enna et al, 1977  Bowen, 1983 Bowen, 1983  <sup>c</sup> Oram et al, 1982 Soininen et al, 1984 Thal et al, 1983 <sup>c</sup> Oram et al, 1981  Soininen et al, 1981a Johnson & Domino, 1971 Wikkelsø et al, 1981 *Wikkelsø et al, 1981 Williams et al, 1980  Alafuzoff et al, 1983 <sup>c</sup> Wikkelsø et al, 1981 Jonker et al, 1982  Shore & Wyatt, 1983	Appleyard et al, 1983 <sup>a</sup> Soininen et al, 1984 Davies, 1979 <sup>a</sup> Wood et al, 1982 <sup>c</sup> Deutch et al, 1983
AChE reduced		
AChE normal		
BChE normal		
b) Reduced AChE:BChE ratio		
c) Normal levels of ChAT		
d) Negative correlation of ACh & degree of dementia		
e) Choline concentration reduced or normal		
normal		
<u>2. Noradrenergic related parameters</u>		
a) MHPG levels normal		Wood et al, 1982
b) DBH activity reduced		
<u>3. Serotonergic related parameters</u>		
5-HIAA levels:		
reduced		Soininen et al, 1981b
normal		Wood et al, 1982
<u>4. Dopaminergic related parameters</u>		
HVA levels:		
reduced		Soininen et al, 1981b
normal		Parkes et al, 1974 <sup>c</sup> Mann et al, 1981 Zimmer et al, 1984
<u>5. Amino acid levels</u>		
a) GABA; reduced		Zimmer et al, 1984
b) aspartate and glutamate; normal		
c) glycine reduced		
<u>6. Neuropeptide levels</u>		
a) Somatostatin:		
reduced		Wood et al, 1982
normal		
b) TRH & LHRH levels normal		
<u>7. Proteins</u>		
a) Total protein:		
reduced		Appleyard et al, 1983 Davies, 1979 Arendt et al, 1984
normal		
b) Abnormal iso-electric focussing & gamma globulin electrophoresis		
c) Albumin:		
increased		
normal		
d) IgA, D, E, G & M normal		Alafuzoff et al, 1983
<u>8. Others</u>		
Normal aluminium levels		

<sup>a</sup> Although these reports failed to make a distinction between AChE and BChE, it is assumed that under the assay conditions used the majority of activity is due to AChE (Davies, 1979)

<sup>b</sup> No distinction made between AChE and BChE and since method different from that of Davies (1979), the majority of activity cannot be assumed to be due to AChE

<sup>c</sup> Not age-matched

<sup>d</sup> Activity expressed per mg protein rather than per ml CSF

\* Dementia not specifically stated as due to SDAT

TABLE 25

Measurement of various biochemical parameters in the blood of SDAT subjects

Observation	References	
<u>1. Cholinergic related parameters</u>		
a) Erythrocyte AChE reduced normal	Chipperfield et al, 1981 Markesbury et al, 1980	Smith et al, 1982
b) Erythrocyte choline uptake normal	Yates et al, 1980	
c) Plasma BChE increased	Smith et al, 1982	
<u>2. Noradrenergic related parameters</u>		
Serum DBH reduced	Miyata et al, 1984	
<u>3. Monoamine oxidase activity</u>		
increased increased per platelet normal Vmax and Km	*Alexopoulos et al, 1984 Adolfsson et al, 1980 Mann et al, 1981	
<u>4. Serum/plasma proteins</u>		
a) Immunoglobulins Ig A: increased normal	*Kalter & Kelly, 1975 Mayer et al, 1976 Pentland et al, 1982 Jonker et al, 1982	Tavolata & Argentiero, 1980 Alafuzoff et al, 1983
Ig D: normal	Kalter & Kelly, 1975 Jonker et al, 1982	Tavolata & Argentiero, 1980 Pentland et al, 1982
Ig E: normal	Jonker et al, 1982	Pentland et al, 1982
Ig G: increased normal	Henschke et al, 1979 Kalter & Kelly, 1975 Tavolata & Argentiero, 1980 Pentland et al, 1982	*Mayer et al, 1976 Jonker et al, 1982 Alafuzoff et al, 1983
Ig M: reduced increased normal	Tavolata & Argentiero, 1980 *Kalter & Kelly, 1975 Mayer et al, 1976 Jonker et al, 1982	Henschke et al, 1979 Alafuzoff et al, 1983
b) Albumin: reduced normal	Behan & Feldman, 1970 Alafuzoff et al, 1983	
c) Complement, C3 & C4: normal	Tavolata & Argentiero, 1980	Pentland et al, 1982
<u>5. Lymphocytes</u>		
B lymphocytes reduced T lymphocytes normal	Tavolata & Argentiero, 1980 Henschke et al, 1979	Tavolata & Argentiero, 1980
<u>6. Erythrocyte parameters</u>		
Altered physical state of membrane proteins	Markesbury et al, 1980	
Increased lithium-sodium countertransport	Diamond et al, 1983	
Normal; SDS-electrophoresis, sodium-potassium ATPase	Markesbury et al, 1980	
<u>7. Others</u>		
Reduced plasma growth hormone during sleep	Davis et al, 1982a	
Reduced response of polymor- pho-nuclear leukocytes to temperature in the presence of SDAT sera	Maysuyama & Fu, 1983	
Normal serum aluminium levels	Shore & Wyatt, 1983	

\* Dementia not specifically stated as due to SDAT



normal and SDAT patients (Parkes et al, 1974; Bowen et al, 1981; Mann et al, 1981; Wood et al, 1982). In addition monohydroxyphenylglycol (MHPG), the metabolite of noradrenaline, has also been examined and appears to be at normal levels (Mann et al, 1981; Wood et al, 1982, Palmer et al, 1984) whilst the levels of the noradrenergic marker enzyme dopamine- $\beta$ -hydroxylase have recently been reported to be reduced (Miyata et al, 1984) in the CSF of SDAT patients. In addition to the classical serotonergic, dopaminergic and noradrenergic neurotransmitter systems, the levels of somatostatin, which, to date, is the only neuropeptide that has been consistently reported to be involved in SDAT (chapter 1, section I.7.b.iii), have also been measured in SDAT patients and both normal (Thal et al, 1983) and decreased (Oram et al, 1981; Wood et al, 1982; Soininen et al, 1984) levels have been reported.

As a possible index of the central nervous amino acid transmitters, the amounts of free amino acids have been measured in the CSF of SDAT patients, and whilst glutamate and aspartate levels did not differ significantly from the normal (Bowen, 1983), decreased levels of glycine were observed (Bowen, 1983). Furthermore, the observation that two out of the three individuals suffering from SDAT examined by Enna et al (1977) had levels of GABA well below normal has recently been repeated in a larger series of patients (Zimmer et al, 1984). However, the interpretation of data concerning amino acids in the CSF is complicated by difficulties in distinguishing neurotransmitter and metabolic pools of amino acids and by the relative lack of data implicating the amino acid neurotransmitter systems in SDAT (chapter 1, section I.7.b).

In contrast, the involvement of the central cholinergic system in SDAT is now well established (chapter 1, section I.7.a.i) and consequently parameters associated with this neurotransmitter system have been more extensively studied in SDAT than the above mentioned systems. Most studies have concentrated on the levels of cholinesterases in the CSF and although several reports have failed to make the distinction between AChE and BChE, it is assumed that under the assay conditions used, the interference due to BChE is negligible (see footnote, table 24). Thus, levels of AChE activity have been reported to be normal (Johnston and Domino, 1971; Davies, 1979; Wood et al, 1982) or decreased (Soininen et al, 1981a; Appleyard et al, 1983; Arendt et al, 1984) per unit volume of CSF. However Soininen et al (1981a) and Appleyard et al (1983) also observed that, in addition to AChE, protein levels were also significantly decreased and consequently when AChE activity was



expressed per unit of protein, the levels of AChE in the normal and SDAT CSF were not significantly different. Other cholinergic-related parameters measured in the CSF of SDAT patients include ChAT (Johnson and Domino, 1971; see, however, Aquilonius and Eckernas, 1976) and choline which have both been reported to be normal (Yates et al, 1980; Growdon and Logue, 1982) and ACh which has been reported to be negatively correlated with the degree of dementia (Johns et al, 1983). However, the amounts of ACh are very small (approximately 40pmol/ml) and since CSF AChE is present at levels of activity of approximately 20nmol/min/ml the physiological significance of the CSF ACh remains unclear.

The protein present in the CSF of SDAT patients appears to contain normal levels of immunoglobulins (Jonker et al, 1982; Alafuzoff et al, 1983) whilst levels of albumin have been reported to be either increased (Alafuzoff et al, 1983) or normal (Wikkelsø et al, 1981). Moreover, total levels of CSF protein have been reported to be both unaltered (Johnson and Domino, 1971; Davies, 1979; Wikkelsø et al, 1981; Arendt et al, 1984) and decreased (Soininen et al 1981a; Appleyard et al, 1983) and this, as illustrated above for the levels of AChE activity, complicates the interpretation of the results obtained in the study of the CSF in SDAT.

In addition to the CSF, blood has also been subject to examination in the search for an SDAT-specific biochemical marker (table 25). In spite of a lack of evidence demonstrating an immunological involvement in the disease process, various groups have examined the levels of immunoglobulins in the plasma or serum of patients suffering from SDAT. However, whilst elevated IgG levels were reported in late-onset SDAT (Henschke et al, 1979), levels essentially within the normal range (Mayer et al, 1976; Tavolato and Argentiero, 1980; Jonker et al, 1982, Alafuzoff et al, 1983) or in the lower normal range (Pentland et al, 1982) have also been reported.

Furthermore, various other parameters not related to any known pathological process have been found to be altered in SDAT blood and include alterations in the physical state of the erythrocyte membrane (Markesbury et al, 1980) erythrocyte countertransport (Diamond et al, 1983), the presence, in sera, of an inhibitor of in vitro leukocyte mobility (Matsuyama and Fu, 1983) and reduced levels of plasma growth hormone during sleep (Davis et al, 1982a). Whilst such reports are of interest, they are unsubstantiated and do not provide a rational basis for a diagnostic test. In contrast, since the central cholinergic system



is severely reduced in SDAT patients, there is a rational reason for studying the cholinesterases in the blood of SDAT patients. There have, however, been relatively few reports concerning the levels of blood cholinesterase activities in SDAT. Furthermore, in those studies in which erythrocyte AChE has been measured as a peripheral marker of SDAT, the data is conflicting. Thus, whilst Chipperfield et al (1981) observed a significant reduction in activity, Markesbery et al (1980) and Smith et al (1982) reported essentially normal levels. Interestingly, erythrocytes also appear to have normal levels of another cholinergic-related parameter, namely choline uptake (Yates et al, 1980). In addition to the levels of erythrocyte AChE, Smith et al (1982) also measured BChE in the plasma of SDAT patients and observed a substantial (100%) and significant elevation of activity above the normal. The present study was therefore undertaken to further examine both erythrocyte AChE and plasma BChE in SDAT patients. Furthermore, AChE has only recently been described in the human plasma (Sketelj and Sasel, 1980; Festoff and Fernandez, 1981; Hodgson and Chubb, 1982; Rasool et al, 1983; Sketelj et al, 1983) and therefore levels of this enzyme, which have not previously been measured in SDAT, were also examined as a possible marker of the central cholinergic deficit. Since plasma AChE activity was the only one of the three cholinesterase activities examined that was altered in the SDAT patients compared to both normal and depressed subjects, activity of this enzyme was also measured in a series of patients suffering from Parkinson's disease which, like SDAT, has also been reported to involve a central cholinergic deficit (Ruberg et al, 1982; Dubois et al, 1983; Perry et al, 1983b). In addition, three blood samples were obtained from subjects suffering from motor neuron disease in which plasma AChE has been previously reported to be elevated above the normal (Festoff and Fernandez, 1981; Rasool et al, 1983).

As well as measuring cholinesterase levels in different clinical groups, plasma AChE was characterised, along with both erythrocyte AChE and plasma BChE, with respect to electrophoretic mobility, sedimentation velocity and substrate affinity (Km). Whilst these particular experiments were carried out primarily to differentiate the plasma AChE and BChE enzymes, comparisons of substrate affinity and electrophoresis were made between blood from normal, depressed and SDAT subjects. A preliminary report of some of the results presented here has been published (Perry et al, 1982c).

## II. MATERIALS AND METHODS

### 1. Subjects

Patients included in this study were all either attending or were resident in the psychogeriatric and geriatric hospital units in Newcastle upon Tyne. Blood was collected on three separate occasions from different patients (series 1, 2 and 3). Patients with psychiatric illness had all received a standardised multidisciplinary psychogeriatric assessment and, using criteria outlined by Roth (1955) and Blessed et al (1968) - chapter 2, section II.1 - were divided into SDAT, endogenous depression, neurotic depression and miscellaneous groups. The miscellaneous group consisted of patients suffering from multi-infarct dementia, hypomania, paranoid psychosis, schizophrenia and acute confusion. The multi-infarct dementia patients constituted the largest proportion of this group (45% of the total series 1,2 and 3 miscellaneous group). The control group consisted of geriatric subjects who had been assessed to confirm the absence of any significant psychiatric disorders.

The blood samples from series 1 were assessed for all three parameters i.e. erythrocyte AChE and plasma AChE and BChE, whilst those from series 2 and 3 were assayed only for plasma AChE. This accounts for the different group sizes shown in table 26, which also shows the ages and sexes of the different groups. Additionally, several blood samples were haemolysed and the plasma from these subjects was assayed for AChE but not included in the pooled data for the subjects' appropriate clinical group.

In addition to the blood samples taken from SDAT patients and other age-matched subjects, further blood samples were removed, for plasma AChE measurements, from patients showing the classical features of Parkinson's disease (tremor and akinesia) without accompanying dementia. Since these patients were from a younger age group than those used in comparison with the SDAT subjects, blood was also removed from a fresh set of younger age matched control subjects all of whom showed no signs of psychiatric disorders. In addition, three blood samples were obtained from patients suffering from motor neuron disease.



TABLE 26

Analysis of patients used in the present study <sup>a</sup>

	Enzyme assay					
	Erythrocyte AChE and Plasma BChE			Plasma AChE		
	Cases	Age years	Sex; M/F	Cases	Age years	Sex; M/F
Neurotic depression	9	74.5 $\pm$ 4.0	2/7	11	76.6 $\pm$ 3.6	2/9
Endogenous depression	9	79.6 $\pm$ 6.0	2/7	13	78.6 $\pm$ 5.4	4/9
Miscellaneous	9	77.0 $\pm$ 7.2	2/7	17	77.8 $\pm$ 5.7	5/12
SDAT	18	77.6 $\pm$ 7.3	4/14	31	79.0 $\pm$ 6.4	8/23
Controls		Not assayed		7	77.7 $\pm$ 4.7	4/3
Haemolysed blood samples		Not assayed		5	74.1 $\pm$ 6.6	1/4
Parkinsons disease		Not assayed		12	69.3 $\pm$ 8.4	9/3
Motor neuron disease		Not assayed		3	61.1 $\pm$ 9.8	3/0
Controls		Not assayed		12	67.5 $\pm$ 8.7	7/5

<sup>a</sup> Values shown are mean  $\pm$  standard deviation

## 2. Blood handling procedures

Approximately 5ml of blood were taken from the arm by venopuncture and placed in heparinized tubes and gently mixed. The tube was then placed on ice for up to five and a half hours before centrifugation at 1000g for 10 mins at 4°C. The plasma layer was transferred to a separate tube whilst the upper 'buffy' coat of white blood cells was removed from the top of the erythrocytes and discarded. The packed erythrocytes and plasma were then stored separately at -20°C for approximately six months until assayed. At this temperature, plasma BChE has been reported to retain more than 95% of its original activity after being stored for three years (see Brown et al, 1981) and erythrocyte AChE is also stable for several years (see Braid and Nix, 1973).

## 3. Chemicals

The majority of chemicals used in the present experiments were purchased from the Sigma Chemical Company (Poole, England) including; ammonium persulphate, bromophenol blue, glycine, physostigmine sulphate, potassium ferricyanide, sodium citrate and N,N,N',N'-tetramethylethylenediamine (TEMED). Acrylamide and N,N-methylene bis acrylamide (both Electran grade), hydrochloric acid (AnalaR grade), formaldehyde (AnalaR grade) and sodium hydrogen malate were all purchased from BDH chemicals (Poole, England), Lubrol WX was obtained from Kodak Laboratories (London, England) and tetraisopropylpyrophosphoramidate (iso-OMPA) was supplied by Koch-Light (Haverhill, England). The source of chemicals used for the enzymatic determinations and the sucrose density gradient analyses were as described in chapters 2 and 3 respectively. The sources of other chemicals used in the present experiments but not stated above can be found in the corresponding sections of chapters 2 and 3.

## 4. Enzyme assays

Enzyme assays were carried out spectrophotometrically using the colorimetric thiocholine method of Ellman et al (1961) in which the rate of production of the yellow, 5-thio-2-nitrobenzoic acid anion (chapter 2,



section II.6) was followed by measuring the increase in absorbance at a wavelength of 412nm using a recording spectrophotometer. Reactions were all carried out at 37°C in 0.1M phosphate buffer, pH 8.0 in a total volume of 3.0ml. In order to allow the inhibitors to inactivate the appropriate enzyme, a 30 minute pre-incubation with the specific inhibitors described below was carried out prior to addition of the substrate.

a) Erythrocyte acetylcholinesterase

Erythrocytes were thawed and 100 volumes of 0.1M phosphate buffer pH 7.4 containing 0.1% (v/v) Triton X-100 were added. The suspension was then gently mixed and placed on ice for 30 minutes. Following this, the suspension was vortexed vigorously and 100µl of the resulting clear lysate were assayed in the presence of the inhibitors described below using  $0.5 \times 10^{-3}$ M acetylthiocholine iodide as substrate.

b) Plasma butyrylcholinesterase

The thawed plasma was vortexed vigorously and then diluted by the addition of 50 volumes of 0.1M phosphate buffer, pH 7.4. Following this, 100µl of the diluted plasma were assayed in the presence of the inhibitors described below using  $0.25 \times 10^{-3}$ M butyrylthiocholine iodide as substrate.

c) Plasma acetylcholinesterase

The thawed plasma was vortexed vigorously and diluted in 4 volumes of the Lubrol WX detergent-containing buffer as described by Festoff and Fernandez (1981). Following this, 100µl of each diluted plasma were assayed in the presence of the inhibitors described below using  $0.25 \times 10^{-3}$ M acetylthiocholine iodide as substrate. To standardize each batch of plasma assayed, aliquots of the same 1% w/v homogenate of whole mouse brain, stored at -20°C, were used as inter- and intra-assay controls. The coefficient of variation of the assay procedure was 7.6% inter-assay and 3.9% intra-assay.

d) Use of inhibitors

Since the erythrocytes were not washed during the initial separation of blood cells and plasma, significant amounts of plasma were presumably present in the erythrocyte fraction. Therefore, in order to inhibit hydrolysis of acetylthiocholine due to BChE present in the plasma,

erythrocyte AChE was assayed in the presence of  $10^{-5}$ M tetraisopropylpyrophosphoramidate (iso-OMPA) - a BChE inhibitor. In the plasma fraction itself, BChE was assayed in the presence of  $10^{-5}$ M 1:5-bis (4-allyldimethylammoniumphenyl) pentan-3 one dibromide (BW284c51), an AChE inhibitor. In the measurement of both erythrocyte AChE and plasma BChE, each sample was also assayed in the presence of  $10^{-5}$ M physostigmine sulphate (eserine), a total cholinesterase inhibitor, which served as a blank correction for non-cholinesterase catalysed substrate hydrolysis.

In the plasma, the measurement of AChE is complicated by the presence of high levels of BChE activity, for although the substrate used in the plasma AChE assay, acetylthiocholine iodide, is hydrolysed more rapidly by AChE than BChE, the relatively much larger quantities of BChE in plasma result in a significant hydrolysis of the substrate. In order to reduce this interference, plasma AChE was therefore assayed in the presence of  $10^{-4}$ M iso-OMPA - which inhibits the majority of the acetylthiocholine hydrolysis due to BChE - and the remaining activity was assayed in the presence and absence of  $10^{-5}$ M BW284c51. Only that activity that was sensitive to BW284c51 was considered to represent AChE.

## 5. Kinetic properties of blood cholinesterases

In order to study the properties of the blood cholinesterases, each of the three enzymes was assayed under conditions identical to those described above except for variations in the substrate concentration, which ranged from 0.1 to 15 mM. The enzyme activity was then plotted as a function of the negative  $\log^{10}$  of the substrate concentration and using such a plot the substrate inhibition properties of the enzyme become apparent. In addition, the reciprocal of the enzyme activity was plotted as a function of the reciprocal of substrate concentration (Lineweaver-Burk plot). Assuming that the enzyme obeys Michaelis-Menton kinetics (in which case the Lineweaver-Burk plot is linear), the intercept on the abscissa gives a value, the Michaelis constant ( $K_m$ ), that is a measure of the affinity of the enzyme towards any particular substrate. Thus, the lower the  $K_m$ , the greater the affinity of the enzyme for the particular substrate being used. This value is therefore a characteristic feature of that enzyme under the conditions used for the enzyme assay. Furthermore, in order to determine whether or not there might be qualitative differences between the blood cholinesterases in the



different clinical groups, the  $K_m$ 's were calculated for blood samples from control, SDAT and depressed subjects.

## 6. Electrophoretic identification of blood cholinesterases

In order to further characterise the blood cholinesterases and to try and ascertain whether plasma AChE has a different mobility to both plasma BChE and erythrocyte AChE, samples of both erythrocyte lysate and plasma were electrophoresed and stained histochemically for cholinesterase activity. The method of polyacrylamide gel electrophoresis was essentially that of Clarke (1964) with the exception that in the present experiments, electrophoresis was carried out in the presence of 0.1% Triton X-100. The gels (final acrylamide concentration 6%) were prepared in 40ml volumes by the addition of the following: i) 2 volumes of an acrylamide stock; 24.0g acrylamide monomer plus 0.64g N,N'-methylene bis acrylamide in 100ml final volume; ii) 1 volume of Tris-glycine; 29g glycine, 6.0g Tris, made up to 1 litre final volume with water; iii) 1 volume of 0.28% v/v N,N,N',N'-tetramethylethylenediamine (TEMED) and iv) 4 volumes of 0.14% w/v ammonium persulphate containing 0.2% Triton X-100.

Gels were cast in glass tubes of length 16cm and internal diameter 0.7cm, to a height 2cm from the top of the tube. The top of the gel was overlaid with water and left to set for approximately 2 hours. When the gel had polymerised, the water overlayer was removed and replaced with electrolyte buffer (which was a 1:10 dilution of a stock consisting of 29g glycine and 6.0g Tris, adjusted to pH 8.1 using 1M hydrochloric acid made up to a final volume of 1 litre and containing 1.0% Triton X-100) and the gel was pre-electrophoresed at a current of 1mA/gel for one hour. After this period the samples - 2µl of plasma or 10µl of erythrocyte lysate (1 volume of erythrocytes added to 4 volumes of 0.1M phosphate buffer pH 7.4 containing 0.125% Triton X-100) - were mixed with 25µl glycerol and 10µl 0.5% w/v aqueous bromophenol blue and loaded onto the gels. The gels were then electrophoresed at a constant current of 2mA per gel until the bromophenol blue marker dye approached the bottom of the gel (a period of approximately 3 hours) and the exact distance migrated by the bromophenol blue marker dye was recorded for each individual gel.

After electrophoresis, the gels were removed and placed in 10%

neutral formalin (4% neutral formaldehyde) for one hour to fix the enzymes. Gels were subsequently washed in three changes of distilled water prior to histochemical staining by the method of Karnovsky and Roots (1964). The principle of this method is that thiocholine released by the enzymatic hydrolysis of substrate reduces the ferricyanide ions present in the medium to ferrocyanide. The ferrocyanide ions then react with copper ions to give the insoluble reaction product, copper ferrocyanide which is deposited at the site of the enzyme. The method itself involves the preparation of an incubation medium made by dissolving 50mg of acetyl- or butyryl-thiocholine (for staining AChE and BChE respectively) in 65ml of 0.1M sodium hydrogen maleate buffer (pH 6.0) followed by additions of 5ml 0.1M sodium citrate, 10ml water and 10ml 5mM potassium ferricyanide.

In order to distinguish AChE and BChE activities, gels were stained using  $10^{-5}$ M BW284c51 and  $10^{-4}$ M iso-OMPA as selective inhibitors of AChE and BChE respectively, whilst to confirm that the observed reaction products were indeed due to cholinesterases, additional gels were incubated with  $10^{-5}$ M physostigmine. The gels were preincubated for 30 minutes in incubation medium containing the appropriate inhibitors but minus substrate, followed by a 30 minute incubation at room temperature in the presence of substrate. After the gels had been stained they were soaked in two changes of distilled water at 15 minute intervals and stored in distilled water. The distance migrated by the middle of each band of staining was measured and divided by the distance migrated by the bromophenol blue dye front (the Rf value).

## 7. Analysis of molecular forms of plasma cholinesterases

The molecular forms of plasma AChE were studied by using similar techniques to those described in more detail elsewhere for the analysis of brain material (chapter 3, section II.3).

In brief, 175 $\mu$ l of plasma obtained from three elderly patients (mean age  $\pm$  standard deviation;  $59 \pm 8.1$ ) were mixed with the marker enzymes alcohol dehydrogenase,  $\beta$ -galactosidase and catalase and loaded onto a 10-40% w/w sucrose gradient. Samples were run in a SW65Ti rotor for 17 hours at 4°C at a speed of 55,000rpm (300,000gmax). The gradients were then fractionated into pre-weighed vials and assayed for AChE, BChE and marker enzymes as described previously (section 4).



### III. RESULTS

#### 1. Properties of blood cholinesterases

##### a) Enzyme kinetic properties

Figure 34 shows the effect of increasing substrate concentration on each of the blood cholinesterases in one representative case from each of the three normal, SDAT and neurotic depression blood samples analysed (left-hand side of figure). Also illustrated (right-hand side of figure) are the corresponding Lineweaver-Burk plots of the reciprocal of enzyme activity plotted against the reciprocal of substrate concentration.

The plot of enzyme activity versus substrate concentration clearly shows that both erythrocyte and plasma AChE demonstrate the phenomenon of inhibition of by high ( $>1-2\text{mM}$ ) substrate concentrations. In contrast, plasma BChE showed no such phenomenon using either acetylthiocholine or butyrylthiocholine as substrate and demonstrated a characteristic sigmoidal response to increasing substrate concentration, indicating that the enzyme does not follow Michaelis-Menton kinetics (Main, 1976). Consequently, whilst a  $K_m$  value can be calculated for both erythrocyte and plasma AChE from the enzyme activity at substrate concentrations below those at which inhibition occurs, the deviation from Michaelis-Menton kinetics of plasma BChE means that the Lineweaver-Burk plot is not linear and therefore the  $K_m$  cannot be measured using this method. However, in order to compare the kinetics of plasma BChE in the different clinical groups, the intercept on the abscissa of a line drawn through the reciprocal plots of activity at relatively high ( $>0.66\text{mM}$ ) concentrations (at which the effects due to the cooperative changes suggested by the sigmoidal  $v$ -against- $s$  plot will be least; P. Jones, personal communication) was calculated and is called  $k'$ . Table 27 shows the mean  $K_m$  and  $k'$  values of three blood samples assayed from the normal, SDAT and endogenous depression groups. The results clearly show that not only do both erythrocyte and plasma AChE have very similar  $K_m$ 's of approximately  $2 \times 10^{-4}\text{M}$  but also that there is no obvious difference in the  $K_m$  of either plasma or erythrocyte AChE between the different diagnostic groups. Similarly, the  $k'$  values calculated for plasma BChE did not vary between the different clinical groups indicating that there is no pathological alteration in substrate affinity for any of the blood

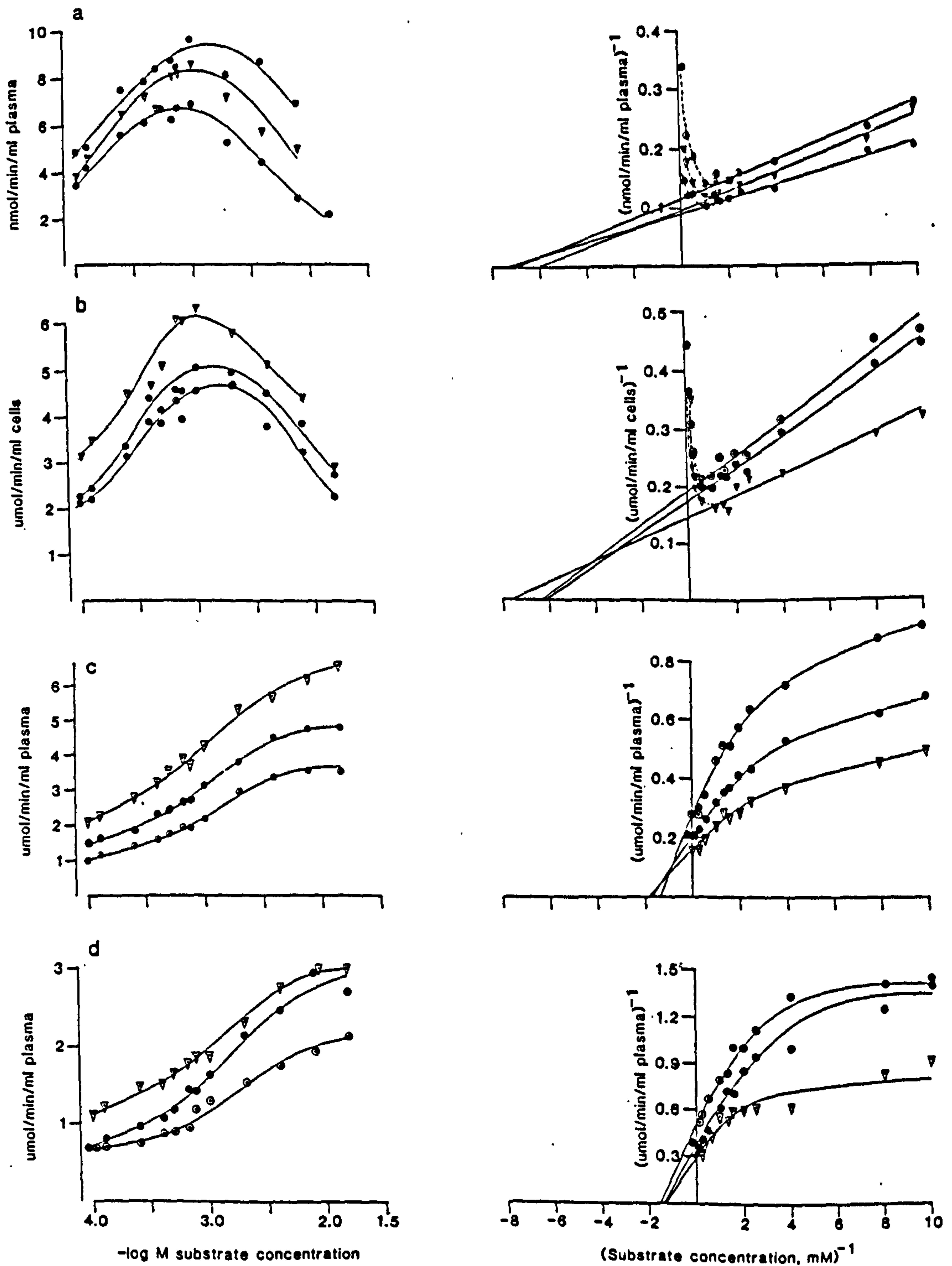


Figure 34. Representative plot of one blood sample out of three assayed from each of the normal, SDAT and endogenous depressive groups. a), plasma AChE; b), erythrocyte AChE; c) plasma BChE, acetylthiocholine substrate; d) plasma BChE, butyrylthiocholine substrate. The plots of enzyme activity versus the negative log of substrate concentration (left hand figures) clearly show presence or absence of substrate inhibition at high substrate concentrations. The Lineweaver-Burk plots (right hand side) allow calculation of the  $K_m$  values for plasma and erythrocyte AChE or  $k'$  values (see text) for plasma BChE. Normal, ●—●; SDAT, ○—○; endogenous depression, ▽—▽.



TABLE 27

Km and k' values ( $\times 10^{-4}$  M) of the different blood cholinesterases<sup>a</sup>

Substrate	Acetylcholinesterase <sup>b</sup>		Butyrylcholinesterase <sup>c</sup>	
	erythrocyte	plasma	plasma	plasma
	AcThChI	AcThChI	AcThChI	BuThChI
Normal	2.49 $\pm$ 0.61	1.88 $\pm$ 1.22	7.13 $\pm$ 0.88	5.49 $\pm$ 0.53
SDAT	1.72 $\pm$ 0.31	1.74 $\pm$ 0.51	6.42 $\pm$ 1.51	6.31 $\pm$ 1.08
Endogenous depression	1.82 $\pm$ 0.46	1.68 $\pm$ 0.16	5.72 $\pm$ 1.46	5.96 $\pm$ 0.82

<sup>a</sup> Values shown are mean  $\pm$  standard deviation (n = 3, except for plasma AChE where n = 4)

<sup>b</sup> Values shown are Km

<sup>c</sup> Values shown are k' (see text)

cholinesterases.

In addition, the  $k'$  values for plasma BChE are very similar whether the substrate is acetylthiocholine or butyrylthiocholine and suggests that, at least at substrate concentrations greater than 0.66mM, BChE has an equal affinity for the two substrates.

b) Electrophoretic separation of blood cholinesterases

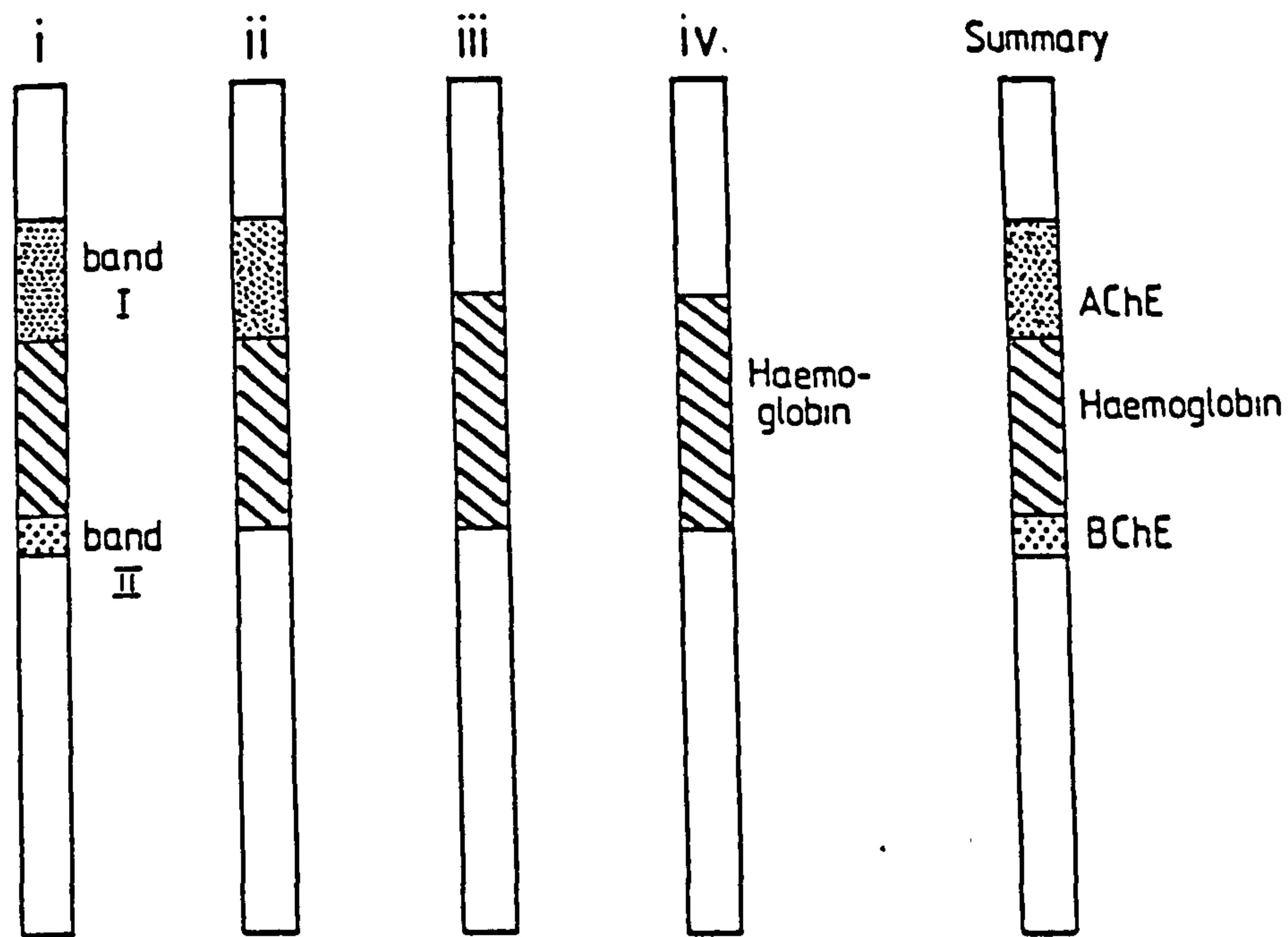
The results of the electrophoretic separation and histochemical localization of enzyme activity are represented diagrammatically in figure 35. Essentially identical patterns were obtained upon electrophoresis of blood samples taken from the different clinical groups analysed and a comparison of  $R_f$  values is shown in table 28.

When erythrocyte lysates were electrophoresed, two opaque brown bands of cholinesterase staining could be identified as judged by their inhibition by  $10^{-5}$ M physostigmine. Thus, in the absence of inhibitors, a broad intensely staining band of activity (band I;  $R_f = 0.23$ ) was observed along with a second less intense band which migrated faster (band II;  $R_f = 0.53$ ). A clear rather than opaque brown region separates bands I and II and is probably due to haemoglobin since the staining in this region is not abolished a mixture of  $10^{-4}$ M iso-OMPA and  $10^{-5}$ M BW284c51 or by  $10^{-5}$ M physostigmine and was also observed to have an identical mobility to the red haemoglobin band observed immediately after completion of electrophoresis. In the presence of iso-OMPA, the activity of band I appears to be unaltered whereas the activity of the more mobile band II was totally inhibited. This suggests that activity in band II was due to the presence of plasma BChE as a contaminant in the erythrocyte fraction of the blood during the initial separation of erythrocytes and plasma. The opaque staining of band I was abolished by  $10^{-5}$ M BW284c51 indicating that it is due to AChE.

The separation of plasma showed, in the absence of inhibitors, three discrete bands of activity, all of which were abolished by  $10^{-5}$ M physostigmine indicating that they were due to cholinesterases. The slowest moving band ( $BC_1$ ;  $R_f = 0.24$ ) constituted the major portion of the activity, whilst the band with the intermediate mobility ( $BC_2$ ;  $R_f = 0.38$ ) showed the weakest reaction and the fastest moving band ( $BC_3$ ;  $R_f = 0.53$ ) had an activity intermediate between the  $BC_1$  and  $BC_2$  bands. In the presence of iso-OMPA, nearly all the plasma cholinesterase activity was abolished except for a small amount which formed a narrow band ( $R_f = 0.27$ ) in the region of the  $BC_1$  band observed in the absence of



## a) Erythrocyte lysate



## b) Plasma

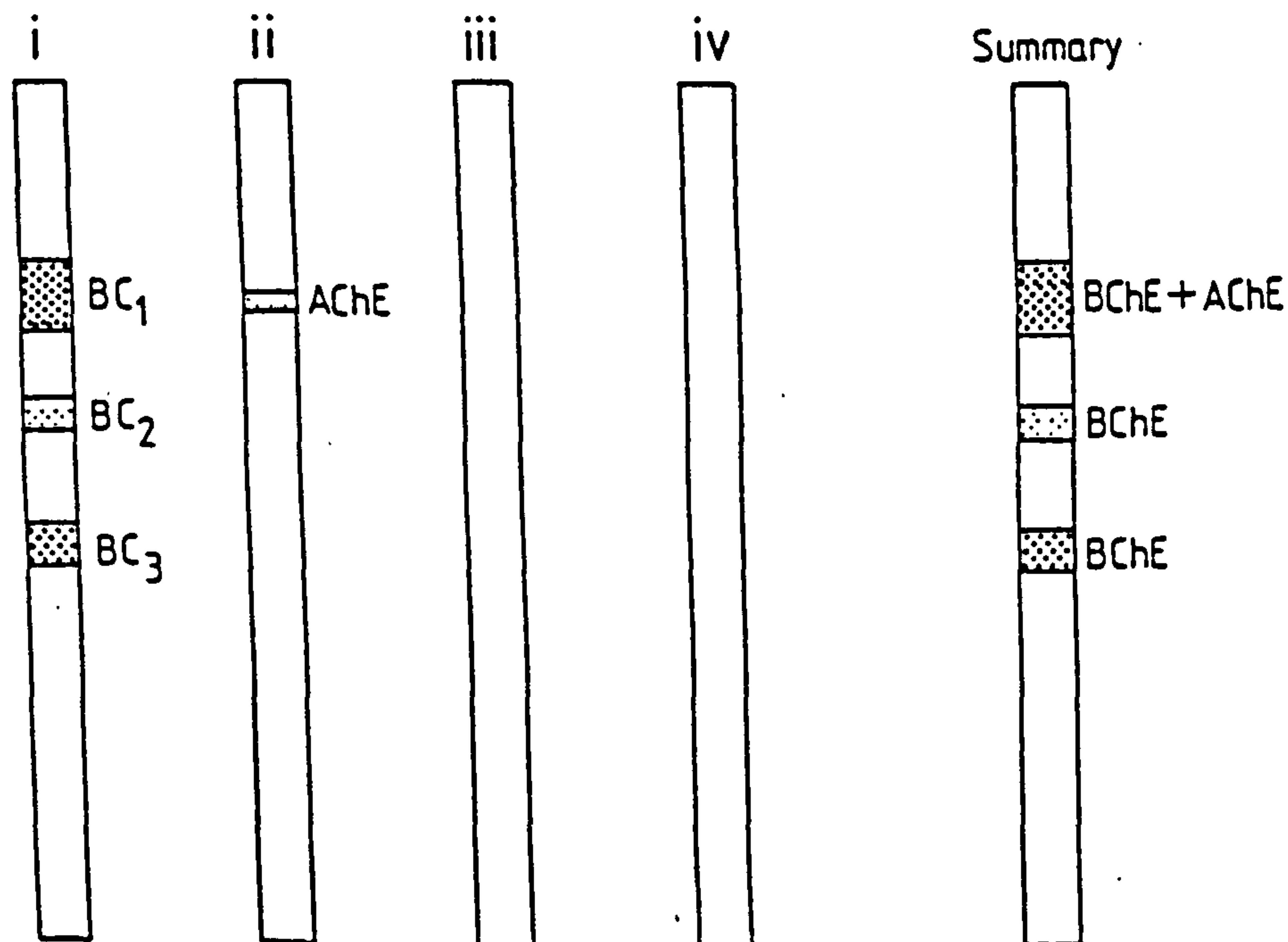


Figure 35. Representative diagram of the electrophoresis of a) erythrocyte lysate and b) plasma from a normal subject, followed by histochemical staining for cholinesterase activity by the method of Karnovsky and Roots (1964). Inhibitor conditions: i, absence of inhibitors; ii, 0.1mM iso-OMPA; iii, 0.1mM iso-OMPA plus 0.01M BW284c51; iv, 0.01mM physostigmine. Density of stippling indicates the relative intensity of cholinesterase staining.

TABLE 28

Comparison of the Rf values of the different forms of blood cholinesterases separated by polyacrylamide gel electrophoresis <sup>a</sup>

ERYTHROCYTE ELECTROPHORESIS				
	Band I (AChE)	Band II (BChE)		
Normal	0.23 ± 0.06	0.53 ± 0.07		
SDAT	0.22 ± 0.04	0.51 ± 0.03		
Endogenous depression	0.19 ± 0.07	0.49 ± 0.10		

PLASMA ELECTROPHORESIS				
	Butyrylcholinesterase			Acetylcholin- esterase
	BC <sub>1</sub>	BC <sub>2</sub>	BC <sub>3</sub>	
Normal	0.24 ± 0.06	0.38 ± 0.09	0.53 ± 0.09	0.27 ± 0.08
SDAT	0.26 ± 0.08	0.35 ± 0.08	0.55 ± 0.11	0.22 ± 0.09
Endogenous depression	0.20 ± 0.08	0.39 ± 0.06	0.52 ± 0.10	0.21 ± 0.07

<sup>a</sup> Values shown are mean ± standard deviation (n = 3, each group)



inhibitors. This band had an intensity of staining roughly equivalent to the intermediate-mobility  $BC_2$  band that stained weakly in the absence of inhibitors. The activity of this iso-OMPA-insensitive band of activity was not abolished by longer preincubations with iso-OMPA, suggesting that it was not due to uninhibited BChE. This activity was, however, abolished by  $10^{-5}M$  BW284c51 indicating that it was probably AChE. Although this plasma AChE band is much narrower than the intense co-migrating  $BC_1$  band of BChE activity, it was not possible to establish whether this band migrated at the front or the rear of the intense co-migrating  $BC_1$  band since, even though gels were formed from the same gel mixture and run at the same time, there was poor inter-gel reproducibility. Thus, although identical samples placed on identical gels had the same number of bands with equivalent relative intensities, corresponding bands did not, however, have identical mobilities and therefore subtle differences in mobility could not be detected. Nevertheless, it was possible to identify the plasma AChE as having a mobility within the range of the  $BC_1$  band of plasma BChE activity and within the broad mobility range shown by erythrocyte AChE; i.e. plasma AChE could not be distinguished electrophoretically from either erythrocyte AChE or plasma BChE.

### c) Molecular forms of plasma cholinesterases

Since plasma AChE could not be distinguished electrophoretically from plasma BChE, sucrose density gradient centrifugation was used in order to try and separate the two enzymes on the basis of their sedimentation velocities rather than their electrophoretic mobilities. The separations of AChE and BChE achieved using three normal plasmas are shown in figure 36, and are analysed in table 29. The mean recovery of activity loaded onto the gradients was 73% for AChE and 79% for BChE and the sedimentation profiles clearly shows that plasma AChE exists as two distinct peaks with sedimentation coefficients of 11.2S and 6.3S respectively, each with approximately equal activities. In addition, BChE also resolved into two regions of activity with the majority (94.5%) of the activity sedimenting as a discrete peak of activity with a sedimentation velocity of 11.8S, whilst a slower-sedimenting zone of activity formed a diffuse band rather than a discrete peak and the midpoint of the band occurred at a point equivalent to a sedimentation velocity of 5.6S.

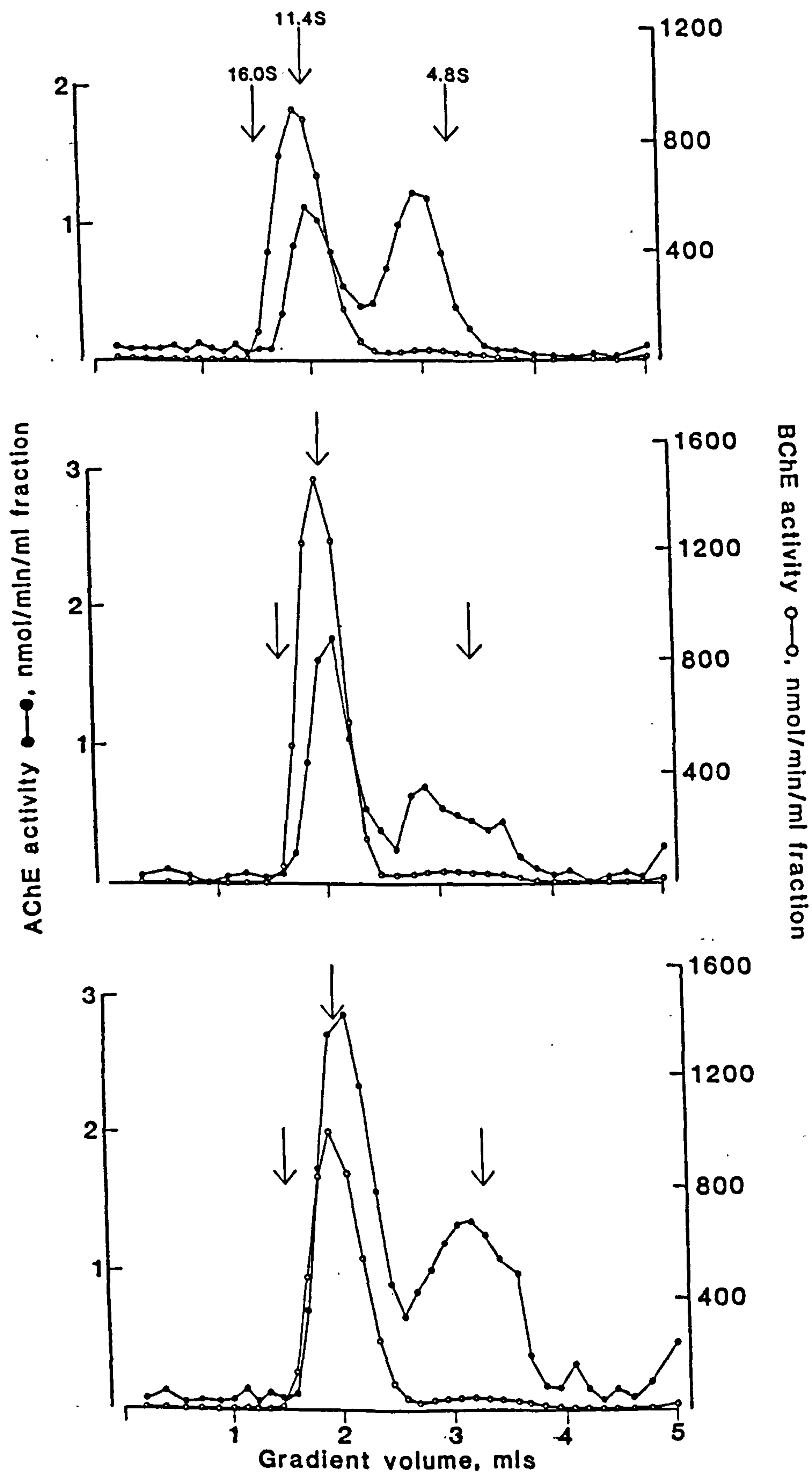


Figure 36. Diagram illustrating the separation of molecular forms of plasma AChE and BChE in three different blood samples taken from normal subjects. AChE, ●—●; BChE, ○—○. Note different scales for AChE and BChE activities. Load = 175  $\mu$ l of each plasma. Sedimentation velocity markers = alcohol dehydrogenase (4.8S); catalase (11.4S);  $\beta$ -galactosidase (16.0S).



TABLE 29

Comparison of sedimentation coefficients and activities of molecular forms of acetylcholinesterase and butyrylcholinesterase in normal plasma <sup>a</sup>

	Plasma AChE		Plasma BChE	
	heavy form	light form	heavy form	light form
Sedimentation velocity <sup>b</sup>	11.18 $\pm$ 0.20	6.27 $\pm$ 0.64	11.75 $\pm$ 0.08	5.63 $\pm$ 0.20
Peak activity <sup>c</sup>	0.915 $\pm$ 0.542	0.712 $\pm$ 0.314	552 $\pm$ 93.4	32.2 $\pm$ 4.9

<sup>a</sup> Results shown are mean  $\pm$  standard deviation (n=3)

<sup>b</sup> Sedimentation velocity is expressed in Svedberg (S) units

<sup>c</sup> Units of activity are nmol/min (derived from 175 $\mu$ l of plasma loaded onto the gradient)

<sup>d</sup> Since the slower sedimenting form of BChE did not separate as a discrete peak of activity, the sedimentation velocity is calculated by determining the position of the mid-point of this region of activity (see text)

## 2. Levels of blood cholinesterases in SDAT: Comparison with other clinical groups

### a) Erythrocyte acetylcholinesterase

Figure 37 and table 30 illustrate the levels of erythrocyte AChE in the different clinical groups of the first series of patients. There was no significant difference between the levels of activity in any of the clinical groups, with the mean activities ranging from 4.32 to 5.31  $\mu\text{mol}/\text{min}/\text{ml}$  packed cells

### b) Plasma butyrylcholinesterase

Figure 38 and table 30 show the levels of plasma BChE in the different clinical groups of the first series of patients. There were no significant differences between the levels of activity in any of the groups, with the mean activities ranging from 4.11 to 4.71  $\mu\text{mol}/\text{min}/\text{ml}$  plasma

### c) Plasma acetylcholinesterase

Figure 39 and table 30 illustrate the data obtained from measurements of plasma AChE in all the patients used in the present study. There was a significant increase ( $P < 0.01$ ) in plasma AChE in the SDAT group of 37% compared with the control group. In addition, plasma AChE in the SDAT group was also significantly ( $p < 0.01$ ) elevated above all other groups including the multi-infarct dementia cases (mean activity;  $6.23 \pm 1.31$ ). Omission of the single very high data point from the SDAT group does not alter the level of significance of these results. In addition, the blood samples which were visibly haemolysed had a mean level of plasma AChE that was not significantly different from any of the other groups. Furthermore, the levels of activity in the Parkinson's disease patients were not significantly different from age-matched control subjects and all three motor neuron disease cases had values that were similar to the mean value of the control group. Because the Parkinson's and motor neuron disease patients were younger than the age-matched SDAT, depressed and miscellaneous subjects (table 26), comparisons were not made between patients from these different age groups.

Since only one of the three cholinesterases examined, namely plasma AChE, provided any evidence of a distinction between SDAT and the other



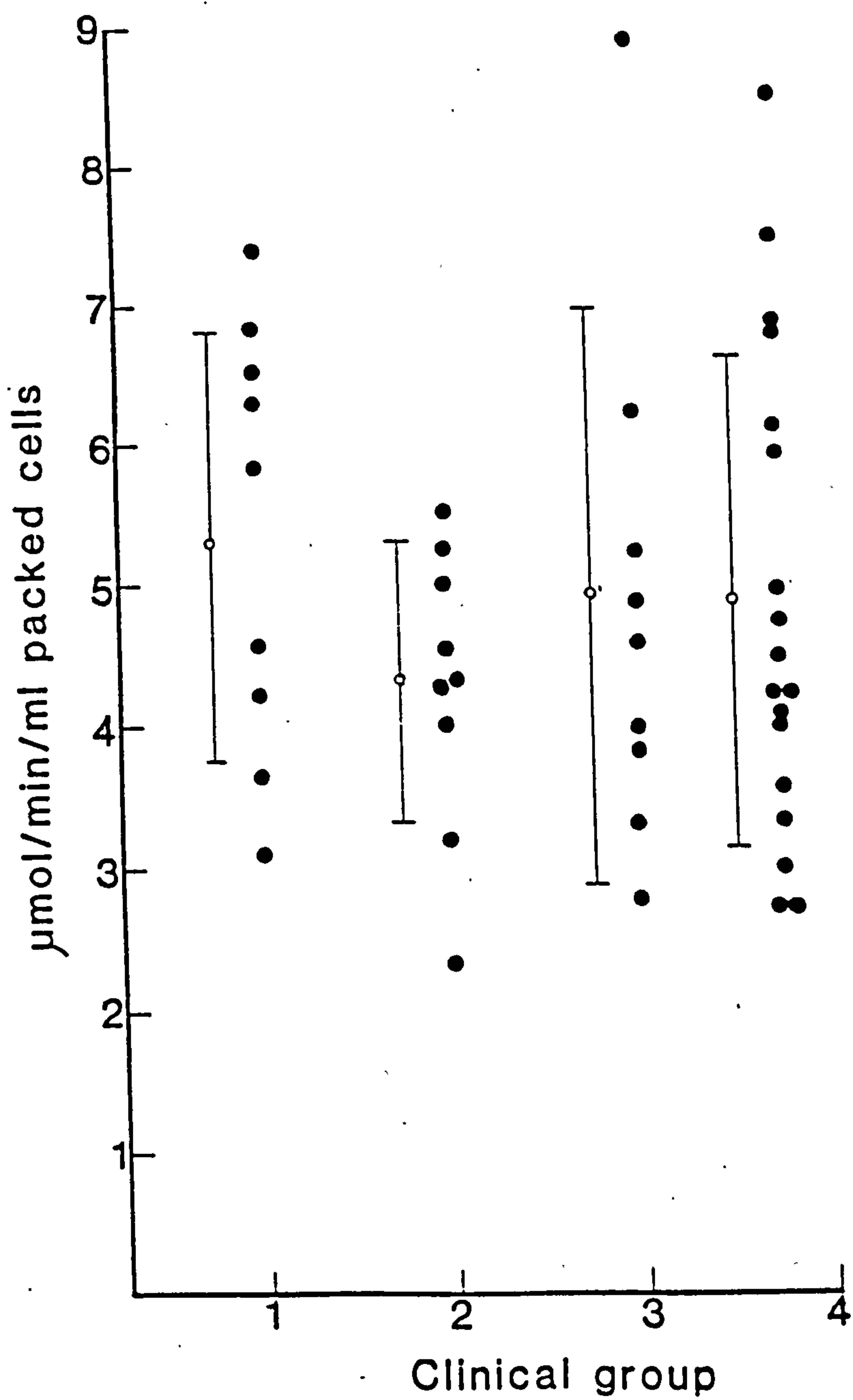


Figure 37. Levels of erythrocyte AChE in different clinical groups. Group 1, neurotic depression; 2, endogenous depression; 3, miscellaneous; 4, senile dementia of Alzheimer type. Vertical bars show standard deviation about the means

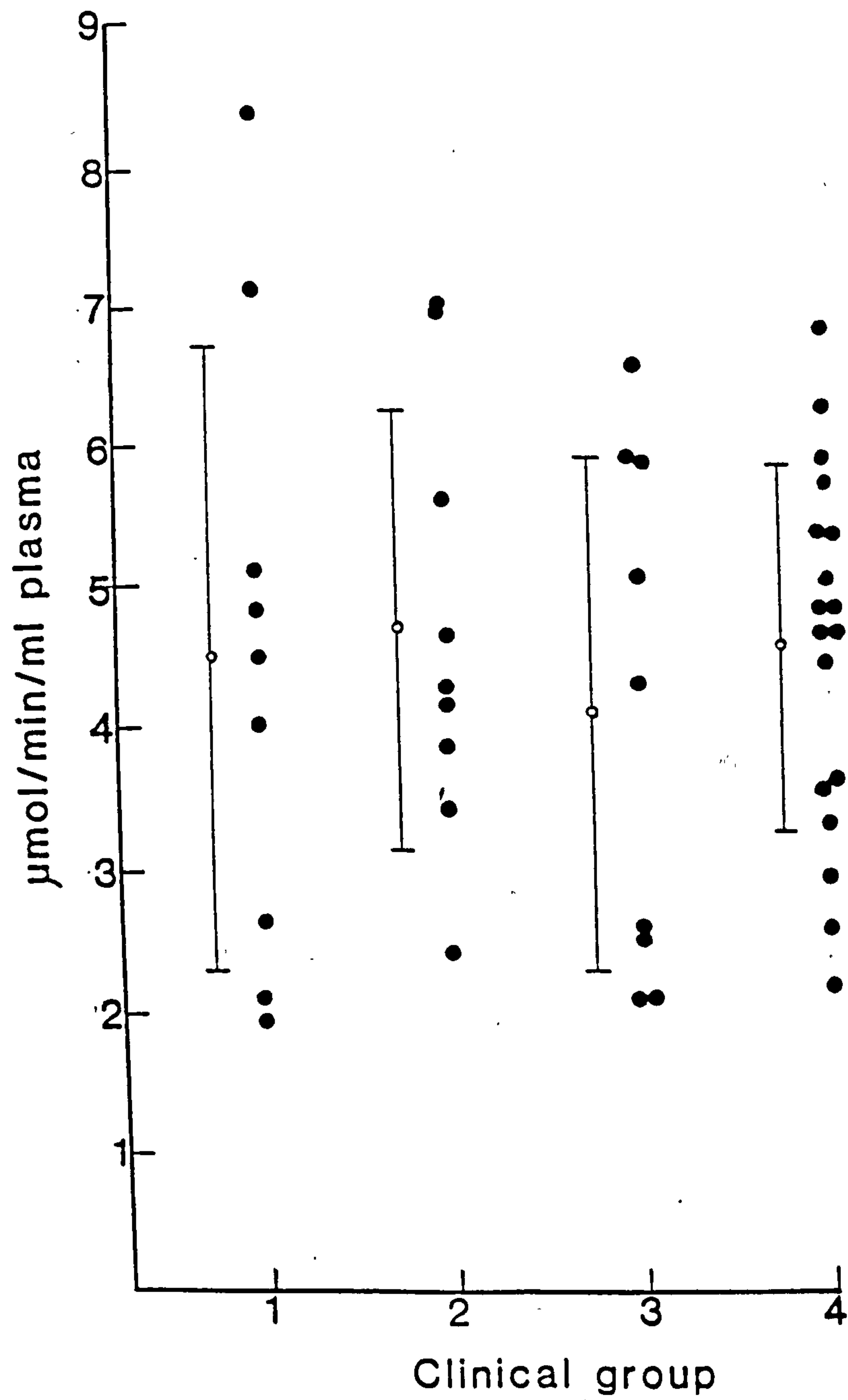


Figure 38. Levels of plasma BChE in different clinical groups. Group 1, neurotic depression; 2, endogenous depression; 3, miscellaneous; 4, senile dementia of Alzheimer type. Vertical bars show standard deviation about the mean.



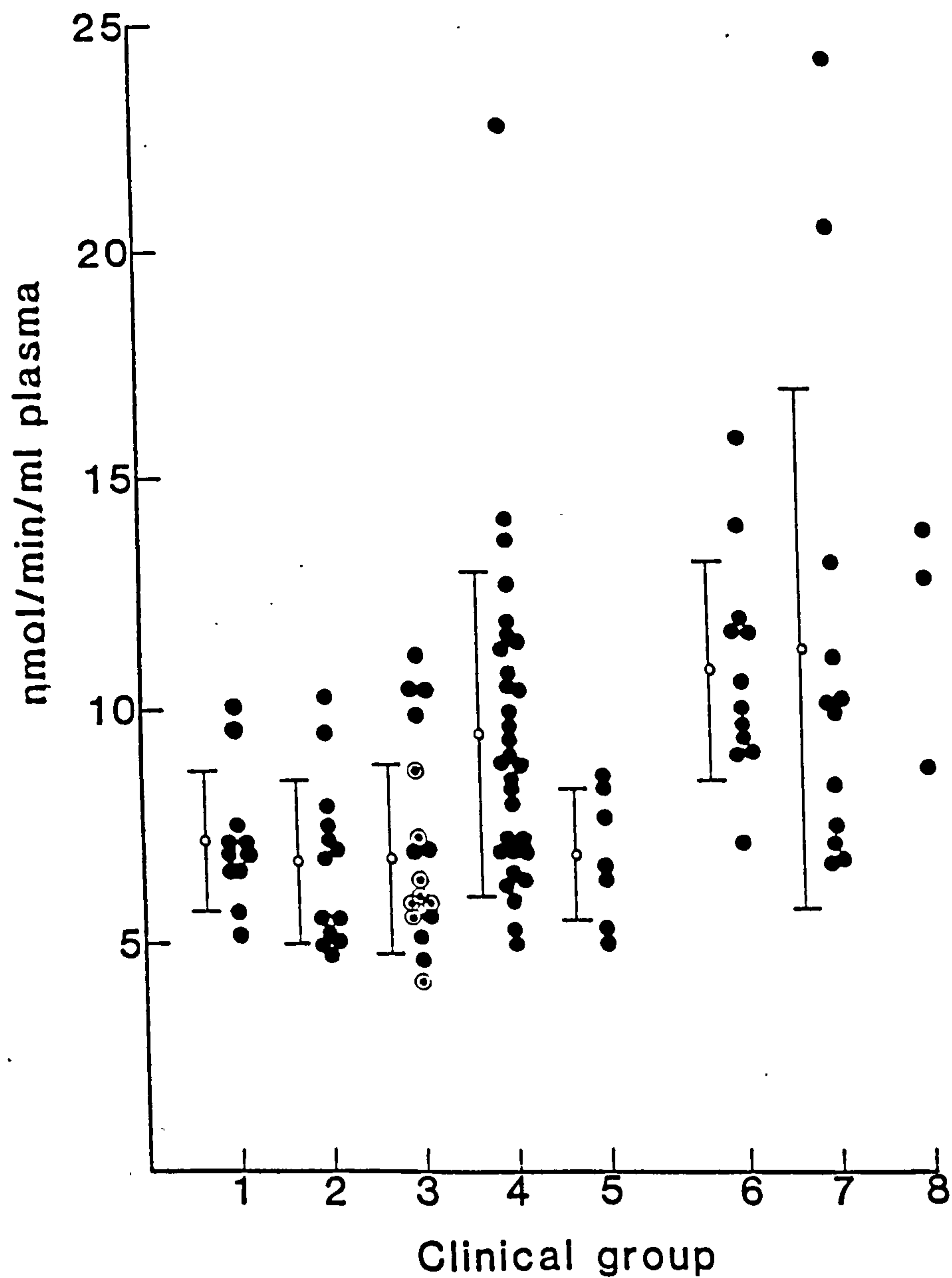


Figure 39. Levels of plasma AChE in different clinical groups. Group 1, neurotic depression; 2, endogenous depression; 3, miscellaneous; 4, senile dementia of Alzheimer type; 5, control; 6, Parkinson's disease; 7, younger controls; 8, motor neuron disease. The miscellaneous group includes cases of multi-infarct dementia (O). Vertical bars show standard deviation about the mean.

TABLE 30

Levels of the different blood cholinesterases in various patient groups <sup>a</sup>

	Erythrocyte AChE	Plasma BChE	Plasma AChE
	$\mu\text{mols/min/}$ $\text{ml packed cells}$	$\mu\text{mols/min/}$ $\text{ml plasma}$	$\text{nmols/min/}$ $\text{ml plasma}$
Neurotic depression	$5.31 \pm 1.54$ (9)	$4.52 \pm 2.20$ (9)	$7.20 \pm 1.48$ (11)
Endogenous depression	$4.32 \pm 1.00$ (9)	$4.71 \pm 1.55$ (9)	$6.76 \pm 1.74$ (13)
Miscellaneous	$4.96 \pm 2.06$ (9)	$4.11 \pm 1.81$ (9)	$6.82 \pm 2.04$ (17)
SDAT	$4.92 \pm 1.75$ (18)	$4.57 \pm 1.30$ (18)	<sup>**</sup> $9.47 \pm 3.47$ (31)
Controls <sup>b</sup>	Not assayed	Not assayed	$6.89 \pm 1.41$ (7)
Haemolysed blood samples	Not assayed	Not assayed	$7.38 \pm 1.81$ (5)
Parkinson's disease	Not assayed	Not assayed	$10.88 \pm 2.40$ (12)
Controls <sup>c</sup>	Not assayed	Not assayed	$11.36 \pm 5.60$ (12)
Motor neuron disease	Not assayed	Not assayed	$11.84 \pm 2.72$ (3)

<sup>a</sup> Data shown is mean  $\pm$  standard deviation. Figures in parentheses show sample size<sup>b</sup> Age-matched with SDAT, depressed and miscellaneous patients<sup>c</sup> Age-matched with Parkinson's disease patients<sup>\*\*</sup> Significantly different ( $P < 0.01$ ) from age-matched neurotic depression, endogenous depression, miscellaneous and control groups.



age-matched clinical groups studied, these data were subjected to an analysis of several nonspecific variables in order to determine whether or not these factors had different influences on the levels of AChE in the plasma of SDAT and non-SDAT subjects. For this purpose, and since plasma AChE activity was not significantly different between the groups, the neurotic depression, endogenous depression, miscellaneous and control values were pooled together. Within neither the pooled groups nor the SDAT group was there any significant difference in activity between male and females. Furthermore, calculation of the regression coefficient of plasma AChE plotted as a function of patient age, time of day of sampling, and delay before storage (table 31) indicated that plasma AChE activity was not associated with age, time of day of blood collection or the interval between collection and storage of plasma. In addition, when the plasma AChE levels of the SDAT group were plotted against the available patient mental test scores (Blessed et al, 1968) there was a trend for the patients with highest mental test scores (i.e. less demented) to have the highest levels of enzyme activity. This trend did not, however, reach levels of significance ( $0.05 < P < 0.1$ ).

TABLE 31

Variation of plasma acetylcholinesterase  
with certain non-specific parameters

Parameter	Age-matched, non-SDAT groups pooled (n=48)		Senile dementia of Alzheimer-type (n=31)	
	Range	Regression coefficient	Range	Regression coefficient
Age	68-89	-0.016	69-89	0.009
Time of day of sampling	10.30- 12.00a.m.	-0.221	10.15- 12.50p.m.	-0.203
Delay before freezing,hrs.	0.5-4.0	0.188	1.5-6.0	0.018

All regression coefficients not significant ( $p>0.1$ )



#### IV. DISCUSSION

Since plasma AChE enzyme has only recently been described in human plasma (Sketelj and Sasel, 1980; Festoff and Fernandez, 1981; Hodgson and Chubb, 1982; Rasool et al, 1983; Sketelj et al, 1983) it remains poorly characterised with respect to both its kinetic and physical properties. Consequently, although the main aim of the present study was to measure the activity of the blood cholinesterases in different clinical groups of geriatric subjects, experiments were carried out to determine the electrophoretic, sedimentation velocity and substrate affinity properties of plasma AChE in comparison to both erythrocyte AChE and plasma BChE. The discussion is therefore divided into two main parts, the first of which discusses the results obtained in the enzyme characterisation experiments and the second discusses the levels of cholinesterases observed in the different clinical groups of elderly subjects.

##### 1. Properties of blood cholinesterases

###### a) Kinetic properties of blood cholinesterases

To date the only evidence suggesting that AChE activity is present in the plasma is the observation that a portion of the plasma choline-ester hydrolytic activity is sensitive to the relatively AChE-specific inhibitor BW284c51 (Festoff and Fernandez, 1981; Rasool et al, 1983; Sketelj et al, 1983). However, this inhibitor is not totally specific for AChE (Silver, 1974) and it may, therefore, be possible that the BW284c51-sensitive activity in the plasma is due to BChE rather than AChE. However, the results presented here demonstrate that a striking feature of the plasma BW284c51-sensitive activity is the inhibition of the enzyme by high ( $>1\text{mM}$ ) substrate concentrations which result in a bell-shaped V-against-S plot. In contrast, plasma BChE demonstrated a characteristic sigmoidal response (Silver, 1974; Main, 1976) to increasing concentrations of both acetyl- and butyryl-thiocholine and showed no inhibition by substrate concentrations as high as  $15\text{mM}$ . Furthermore, not only does the plasma BW284c51-sensitive activity differ from BChE in its behaviour at high substrate concentrations, but it also differs at low ( $<0.5\text{mM}$ ) substrate concentrations in that, unlike BChE, it does not deviate from Michaelis-Menton kinetics. Therefore, since



inhibition by high substrate concentrations is a characteristic feature of AChE but not BChE (Silver, 1974; Main, 1976; chapter 1, section II.5), it would appear that the plasma BW284c51-sensitive activity is indeed due to AChE and is not merely an artefact of the much more abundant BChE.

Having distinguished the plasma AChE and BChE enzymes on the basis of their substrate hydrolysing properties, it is interesting to note that the plasma AChE activity has an affinity for acetylthiocholine ( $K_m$ ) virtually identical to that observed for the erythrocyte AChE. Furthermore, these values ( $K_m = 2 \times 10^{-4}M$ ) agree well with those reported previously, using acetylthiocholine as substrate, for human erythrocytes and also human caudate nucleus extracts (Shafai and Cortner, 1971; Niday et al, 1977, 1980; Sorensen et al, 1982) and values obtained for AChE using acetylcholine as substrate in tissues from various species (Main, 1976). Therefore, this data suggests that not only is the plasma BW284c51-sensitive activity not due to BChE but also that it is very similar to AChE from other sources and confirms more specifically than the observations that the enzyme is sensitive to BW284c51 (Festoff and Fernandez, 1981; Rasool et al, 1983; Sketelj et al, 1983), that human plasma does contain AChE.

#### b) Separation of blood cholinesterases by gel electrophoresis

Having distinguished plasma AChE and BChE using substrate affinity properties, plasma was electrophoresed in order to determine whether or not these molecules had different electrophoretic mobilities. In addition, the mobility of erythrocyte AChE was also determined to see how this compared with the mobility of the plasma AChE.

The present observation that plasma contains three distinct forms of BChE activity differs from the results of Harris et al (1962) who were able to identify four BChE variants. This discrepancy is probably due to the higher resolution, two-dimensional electrophoretic technique used by Harris and colleagues. However, the observation that the slowest migrating of the four forms separated by two dimensional electrophoresis constitutes the majority of the activity (see Silk et al, 1979) is in agreement with the present observation that the least mobile band ( $BC_1$ ) contained the greatest enzyme activity. In addition to BChE, it was also possible to demonstrate AChE activity present in the plasma but a distinction between plasma AChE and BChE could not be made according to electrophoretic mobility since the plasma AChE co-migrated with the slowest-migrating ( $BC_1$ ) band of BChE activity. Similarly, the AChE in



the plasma could not be distinguished electrophoretically from the erythrocyte AChE since the form present in the plasma had a mobility within the broad range observed for the erythrocyte enzyme.

c) Separation of plasma cholinesterases by density gradient centrifugation

In contrast to polyacrylamide gel electrophoresis, it was possible to separate the different plasma cholinesterases using sucrose density gradient centrifugation. Thus, when the enzymes are separated on the basis of their sedimentation velocities rather than their electrophoretic mobilities, both plasma AChE and BChE separated as two distinct regions of activity. However, the distribution of activity between the two peaks of AChE activity was strikingly different from the two peaks of BChE activity. Hence, whilst the faster sedimenting peak constituted the major portion (94.5%) of the BChE activity, the faster sedimenting AChE form constituted 56% of the total. It is also interesting to note that, comparing the faster sedimenting forms of AChE and BChE - which both correspond to  $G_4$  quaternary structures - the BChE form sediments faster than the corresponding AChE form (sedimentation velocities 11.8S and 11.2S respectively) which is in agreement with observations made in the human CNS (chapter 3, section III.2).

The present results obtained for the sedimentation coefficient of the most abundant form of plasma BChE are in good agreement with previous reports (table 32). However, the slower sedimenting form observed in the present results has not always been detected. This discrepancy is presumably due to the fact that in previous reports partial purification has often been carried out prior to centrifugation, whereas in the present study whole plasma was used. In addition, only one slower sedimenting peak of activity was detected in the present study whilst Sketelj and Sasel (1980) were able to distinguish two forms sedimenting slower than the main form. The probable explanation for this discrepancy is that the broad peak detected here probably does contain two discrete forms, similar to those reported by Sketelj and Sasel (1980), which have not resolved, possibly due to the high protein load used, resulting in a broad band of activity.

To date, there have been no reports demonstrating the molecular forms of human plasma AChE although Festoff (1982) - without showing data - discussed the fact that the  $G_4$  form constitutes 60-80% of plasma AChE, which is similar to the levels described in the present report. It would

TABLE 32

Previous reports concerning density gradient  
analysis of human plasma cholinesterases

Investigators	Enzyme	S value (% total activity)	Comments
Present study	AChE	11.2 (56%) 6.3 (44%)	
Festoff (1982)	AChE	11 (60-80%)	No data shown
-----			
Present study	BChE	11.8 (94.5%) 5.6 (5.5%)	
Surgenor & Ellis (1954)	BChE	11.9	
Haupt et al (1966)	BChE	10.7	
Das & Liddell (1970)	BChE	12.4	
Meunch et al (1976)	BChE	11.1 5.0	<sup>a</sup> C <sub>4</sub> form C <sub>1</sub> form (C <sub>2</sub> & C <sub>3</sub> not analysed)
Sketelj & Sasel (1980)	BChE	11.7 (90%) 7.4 }=(10%) 5.4 }	

<sup>a</sup> C<sub>1-4</sub> refer to the four different forms of BChE separated by Harris et al (1962)



therefore appear that with respect to both sedimentation velocity and enzyme kinetics plasma AChE can be distinguished from plasma BChE and it may therefore be concluded that the AChE activity is not merely an artefact of the much more abundant plasma BChE.

## 2. Levels of blood cholinesterases in SDAT: Comparison with other clinical groups

The following part of the discussion will concentrate on the levels of cholinesterase activities observed in the different groups of elderly subjects. Since the only significant change observed was an elevation of plasma AChE activity in the SDAT patients, particular attention will be paid to these results and the possibility that this enzyme may be derived from, and therefore reflect the state of, central cholinergic structures.

### a) Erythrocyte acetylcholinesterase

The levels of approximately  $5\mu\text{mol}/\text{min}/\text{ml}$  packed erythrocytes observed in the present study agree with the observation of Augustinsson (1955) who reported a level of activity of  $2.5\mu\text{mol}/\text{min}/\text{ml}$  erythrocytes. The present results are also in the same range as those reported by Milstoc et al (1975) and Festoff and Fernandez (1981) of  $10\mu\text{mol}$  and  $30\mu\text{mol}/\text{min}/\text{ml}$  respectively, although it should be noted that Mathew et al (1980, 1982) and Smith (1982) reported the phenomenal rates of  $4\text{--}5\text{mol}$  hydrolysed/min/ml.

Since the purpose of this study was to evaluate erythrocyte AChE as a possible SDAT-specific diagnostic marker, a series of miscellaneous psychiatric patients were used as a control group and as such they form no more than a baseline for comparison with the SDAT and depressed patients. The results of the present study showed that the levels of erythrocyte AChE were not significantly different between any of the clinical groups studied and these results are compared with observations made in previous reports in table 33. The use of erythrocyte AChE in the diagnosis of SDAT was suggested by Chipperfield et al (1981) who observed a significant reduction ( $P < 0.01$ ) of erythrocyte AChE in the SDAT group compared with both a miscellaneous group of psychiatric patients and healthy controls. Although values of activity were not given, the reduction appeared to be of the order of 30-35%. However, the levels of erythrocyte AChE were not found to be altered significantly in SDAT

TABLE 33

## Blood cholinesterases in various psychiatric and nonpsychiatric disorders

Observation	References
<u>I) Plasma acetylcholinesterase</u>	
1) Amyotrophic lateral sclerosis increased	Festoff & Fernandez, 1981 Rasool et al, 1983
2) Muscular dystrophy normal	Sketelj et al, 1983
3) Diseases of the neuromuscular junction normal	Rasool et al, 1983
<u>II) Erythrocyte acetylcholinesterase</u>	
1) SDAT reduced normal	Chipperfield et al, 1981 (approx. 30-35%) Markesbury et al, 1980 Smith et al, 1982
2) Depression reduced  normal	Milstoc et al, 1975 (15%) Mathew et al, 1982 (44%) see Domino & Krause, 1972
3) Anxiety normal	Mathew et al, 1982
4) Schizophrenia reduced increased normal	Pandey et al, 1970 (51%) see Domino & Krause, 1972 see Domino & Krause, 1972
5) Epilepsy normal	see Domino & Krause
6) Hirschprung's disease increased	Boston et al, 1978 (90%)
7) Amyotrophic lateral sclerosis normal	Festoff & Fernandez, 1981 Rasool et al, 1983
<u>III) Plasma butyrylcholinesterase</u>	
1) SDAT increased	Smith et al, 1982 (101%)
2) Depression increased  normal	Mathew et al, 1982 (85%) see Domino & Krause, 1972 Milstoc et al, 1975
3) Anxiety increased	Mathew et al, 1980 (45%) see Domino & Krause, 1972
4) Schizophrenia increased, reduced & normal	see Domino & Krause, 1972
5) Epilepsy decreased	see Domino & Krause, 1972
6) Hirschprung's disease increased	Boston et al, 1978 (134%)
7) Others decreased <sup>a</sup> increased <sup>b</sup>	Silk et al, 1979; Brown et al, 1981 Silk et al, 1979; Brown et al, 1981

<sup>a</sup> Includes: liver disease, starvation, burns, cancer and myocardial infarction

<sup>b</sup> Includes: alcoholism, psoriasis, hypertension and obesity



either in the present study or in previous reports (Markesbury et al, 1980; Smith et al, 1982).

A further point of interest arising from the present study is the observation that levels of activity in the depressed patients do not appear to be altered relative to the miscellaneous group. This contrasts with the work of Milstoc et al (1975) and Mathew et al (1982) who observed reductions in erythrocyte AChE of 15% ( $p < 0.001$ ) and 45% ( $p < 0.001$ ) respectively in depressed patients. Although this discrepancy could be due to the fact that in the present report a miscellaneous psychiatric group was used for comparison, this is unlikely since Chipperfield et al (1981) found no significant difference between the activities of a miscellaneous psychiatric group and normal healthy controls. Furthermore, and in agreement with the present observation, normal levels of erythrocyte AChE in depressed subjects have also been reported previously (see Domino and Krause, 1972). The discrepancy between the present results and those of Milstoc et al (1975) and Mathew et al (1982) may be due to the older (mostly >70 years old) depressed patients used in the present study although Milstoc et al (1975) did, however, observe no effect of age on the reductions of activity observed in depressed patients between the ages of 20 and 70. Additional studies of erythrocyte AChE in various psychiatric illnesses have shown the levels to be unaltered in anxiety (Mathew et al, 1980) and significantly reduced in schizophrenia (Pandey et al, 1970) whilst significant increases in activity have been reported in the non-psychiatric disorder of Hirschprung's disease (Boston et al, 1978).

In summary, the present study shows that measurements of total erythrocyte AChE activity cannot distinguish between SDAT and depressed patients and therefore presumably cannot distinguish between dementia and pseudodementia caused by depression - which is a major cause of misdiagnosis of dementia (chapter 1, section I.5.a). This, along with previous reports suggesting that altered levels occur in other disorders, would therefore appear to limit the usefulness of this enzyme as a diagnostic marker for SDAT.

#### b) Plasma butyrylcholinesterase

The activities observed in the present report of approximately  $4 \mu\text{mol/min/ml}$  are in good agreement with those reported by Augustinsson (1955), Milstoc et al (1975), Mathew et al (1980, 1982) Smith et al (1982) and Sketelj et al (1983) who all observed activities in the range



of 2 to 7  $\mu\text{mol/min/ml}$  plasma.

The results obtained in the present report are compared with previous studies in table 33. In the present study levels of plasma BChE were not significantly different between any of the clinical groups studied including SDAT and depressed subjects. In contrast, however, it has been reported that plasma BChE is significantly increased by 100% in SDAT patients (Smith et al, 1982). In addition, elevated levels of activity were observed in depressed patients - in agreement with earlier reports (see Domino and Krause, 1972) - by Mathew et al (1982) who observed an increase in activity of 85% compared to normals. Consequently, increased levels of activity in both the SDAT and depressed groups of 100% and 85% respectively might explain why the two groups could not be distinguished in the present study. However, if this was correct, it would imply that BChE levels in the miscellaneous group, which had levels comparable to the SDAT and depressed groups, were also elevated by 85-100% above normal levels, which would seem unlikely considering the wide range of psychiatric conditions included within this group. It is therefore more likely that the present results reflect normal levels of the enzyme in both SDAT and depression in agreement with the report of Milstoc et al (1975) who also found normal levels in depressed subjects. Whilst there is no obvious reason for the conflicting results of the present report and the studies of Mathew et al (1982) and Smith et al (1982), it is interesting to note that conflicting data also exists for the levels of plasma BChE in schizophrenic patients, with increased, decreased and normal levels all being reported (see Domino and Krause, 1972). Indeed, the conflicting data observed for plasma BChE levels in different psychiatric disorders may be due to the influence of various non-psychiatric associated factors which may lead to either increased or decreased levels being observed. Thus, increased plasma BChE activity has been reported in Hirschsprung's disease (Boston et al, 1978) alcoholism, psoriasis, hypertension and obesity (Silk et al, 1979; Brown et al, 1981), whilst decreased levels of plasma BChE have also been reported to be associated with a wide range of factors including starvation, contraceptive pills, burn injuries, cancer and myocardial infarction (Silk et al, 1979; Brown et al, 1981). In addition, since plasma BChE is produced by the liver, its levels are also reduced in liver diseases such as hepatitis and cirrhosis (Silk et al, 1979; Brown et al, 1981). Indeed, plasma BChE is sometimes used clinically as an index of liver function and has proved helpful in



assessing progress in cases of liver transplant (Silk et al, 1979).

In conclusion, it would appear that the measurement of plasma BChE activity is of limited diagnostic value in SDAT, for not only were the levels of the SDAT and depressive groups indistinguishable, but the enzyme activity is influenced by a wide range of nonpsychiatric-related factors.

### c) Plasma acetylcholinesterase

The levels of plasma AChE observed in the present series of approximately 7nmol/min/ml plasma are of the same order of magnitude as those reported by Hodgson and Chubb (1982) and Sketelj et al (1983) of 4.5 and 1.6 nmol/min/ml respectively. The discrepancy in actual values is probably due to the very different assay procedures and conditions used.

The results of the present study suggest that plasma AChE is elevated in SDAT but not depression, Parkinson's disease or multi-infarct dementia. Furthermore, whilst diurnal fluctuations in plasma AChE have been reported (Hodgson and Chubb, 1982), in the present study no correlation was observed in either the SDAT or non-SDAT groups between plasma AChE activity and the time of day of sampling suggesting that diurnal factors did not influence the present study. Interestingly, the levels of the noradrenergic-associated marker enzyme dopamine- $\beta$ -hydroxylase have, been reported, in contrast to AChE, to be decreased in the serum of SDAT subjects (Miyata et al, 1984).

The interpretation of the elevated levels observed in SDAT depends to a large extent on determining the origin of plasma AChE. It is possible that lymphocytes may be a source of the plasma AChE since they have recently been shown to possess AChE activity (Bartha et al, 1982). However, their activity is of the order of 80nmol/min/ $10^9$  cells and assuming a lymphocyte concentration in whole blood of  $2.0 \times 10^9$  cells/litre, this is equivalent to an activity in whole blood of 0.16nmol/min/ml. Therefore, considering their relatively low activity, it would appear unlikely that lymphocytes contribute significantly to the plasma AChE activity of 7nmol/min/ml.

More significantly, sucrose density gradient centrifugation resolved two peaks of activity, the faster sedimenting one of which, using the nomenclature of Bon et al, 1979 (chapter 1, section II.9), probably represents the tetrameric globular ( $G_4$ ) form, whilst the slower sedimenting peak probably represents a mixture of the dimeric and

monomeric globular forms ( $G_2$  and  $G_1$ ) which have not been resolved. Since human erythrocyte AChE is found to exist as the dimeric ( $G_2$ ) form (Ott et al, 1975, 1982), it may be the source of the  $G_2$  component of the slower sedimenting peak of activity if released from erythrocyte membranes in its native form. Furthermore, the erythrocyte  $G_2$  form may also account for the  $G_1$  form of plasma AChE if the released dimer was subject to proteolysis. Thus, it may be possible that at least part of the plasma AChE is derived from the AChE-rich erythrocytes, particularly since erythrocytes lose activity with age (Silver, 1974; Galbraith and Watts, 1981) and also the plasma AChE had an electrophoretic mobility in the region of the broad erythrocyte AChE band. However, the observation that plasma samples separated from haemolysed blood (caused by erythrocyte disruption during sampling) do not have significantly elevated levels of AChE, suggests that plasma AChE may not be the result of a physically-induced ("wear and tear") release of membrane associated enzyme. Similarly, Galbraith and Watts (1981) observed no spontaneous release of AChE from washings of isolated erythrocytes. Nevertheless, even assuming that AChE was released by erythrocytes into the plasma, it is unlikely that the erythrocyte  $G_2$  form of AChE would be released from the erythrocyte and aggregate to form the tetramer, and therefore it would appear that at least 50% of the plasma AChE (i.e. the proportion of  $G_4$  in total plasma AChE) is derived from non-erythrocyte sources.

The most likely sources of the plasma  $G_4$  AChE are tissues associated with cholinergic neurotransmission such as the brain or muscle. It is therefore interesting to note that both nerve and muscle tissues release soluble AChE, both in vivo and in vitro and that the brain is thought to be the source of soluble AChE found in the CSF (chapter 1, section II.8).

It is therefore conceivable that, since the  $G_4$  form is the major form in human brain (Sorensen et al 1982; chapter 3, section III.2) yet is a quantitatively minor component of the total AChE activity present in human muscle (Carson et al 1979), the brain may be the source of at least a part of the plasma  $G_4$  form. Although this would presumably require some form of transport system across the blood-brain-barrier, it is interesting to note that after injection of the irreversible inhibitor soman into the cat, regeneration of plasma AChE activity paralleled the recovery of soluble brain AChE (Yaksh et al, 1975).

Furthermore, although the sedimentation velocity is greater for the  $G_4$  form of AChE in the plasma (11S) compared to the brain (approximately 10S), this is probably due to the fact that the enzyme in the plasma is



soluble whereas that in the human brain is mainly hydrophobic and membrane bound (Sorensen et al, 1982; chapter 3, section III.2.b) and soluble forms have been reported to sediment faster than their corresponding hydrophobic forms (Lazar and Vigny, 1980; Grassi et al, 1982).

In addition to the present observation that plasma AChE activity is elevated in SDAT, levels have also been reported to be elevated in another disorder of the central nervous system (table 33), amyotrophic lateral sclerosis, in which motor neurons of the spinal cord and skeletal muscle degenerate (Festoff and Fernandez, 1981; Rasool et al, 1983). It is therefore interesting that in the three motor neuron disease cases examined in the present study, a similar elevation of activity was not observed. However, since only three subjects were analysed in the present study, the small sample size does not allow meaningful comparisons to be made between this and previous studies, particularly since normal levels of activity were found in several of the amyotrophic lateral sclerosis patients in the reports of Festoff and Fernandez (1981) and Rasool et al (1983). In contrast to motor neuron disease, Duchenne muscular dystrophy is considered to be primarily a muscular rather than a neuronal degenerative disorder and in this disease plasma AChE levels have been reported to be normal (Sketelj et al, 1983). This observation further supports the concept that increased levels of AChE in human plasma may be derived from nervous tissue. Furthermore, although Festoff (1982) has proposed that the increased plasma AChE found in amyotrophic lateral sclerosis may originate from the neuromuscular junction, Rasool et al (1983) observed no elevation in plasma AChE in diseases of the neuromuscular junction, again suggesting a neuronal source of the additional plasma AChE. Compatible with this hypothesis, the release of AChE from motor neurons is known to occur in vivo (Kreutzberg and Toth, 1974; Kreutzberg et al 1975).

It should be mentioned that in dystrophic chickens there is a significant increase in plasma AChE levels (presumably released from the dystrophic muscle; Wilson et al, 1973; Lyles et al, 1980). Whilst this would indicate that, in the chicken, plasma AChE is probably derived from muscle, extrapolation from the chicken to the situation in humans is difficult for two reasons; i) the distribution of blood cholinesterases vary widely from species to species - for example AChE is almost totally absent from chicken erythrocytes whereas it is abundant in humans (Silver, 1974) - and it is therefore likely that there is a variation

between species in the origin of plasma AChE and ii) unlike the chicken, there was no elevation in human plasma AChE in muscular dystrophy (Sketelj et al, 1983).

The elevated plasma AChE observed in the present series of SDAT patients could be related to at least two cerebral abnormalities i) increased leakage through the blood-brain barrier or ii) increased release of soluble AChE from degenerating cholinergic neurons which might then leak into the plasma. In SDAT, both disturbances of the blood-brain barrier, particularly in the region of the senile plaque (Wisniewski and Kazlowski, 1983) and a degeneration of cholinergic neurons in the nucleus of Meynert and their associated axonal processes (chapter 1, section I.7.a.i) occurs. It is therefore possible that either or both these mechanisms may occur in SDAT, particularly in the region of the senile plaque where, in addition to blood-brain-barrier disturbances, neurite degeneration has also been reported to occur (chapter 1, section I.6.b). Furthermore, in Parkinson's disease - in which, like SDAT, there is a central cholinergic deficit (Ruberg et al, 1982; Dubois et al, 1983; Perry et al, 1983) - no alterations in plasma AChE levels were observed, and it is therefore possible that the characteristic neuropathological changes associated with SDAT play a more important role in the elevated levels of plasma AChE than the degeneration of cholinergic structures per se.

Since only approximately 25% of the SDAT cases have a plasma AChE activity above the normal range, the measurement of total plasma AChE activity would seem to be of limited usefulness as a diagnostic marker of moderate to severe SDAT. The observation of a trend for the less severely demented patients to have higher levels of activity suggests that in the early stages of the disease, presumably when the most important pathological changes are occurring, plasma AChE may be elevated above normal to a greater extent than observed here for moderate to severe SDAT cases. Thus, the measurement of levels of plasma AChE in SDAT may be of more use as a diagnostic parameter in the early stages. To test this hypothesis, and since it is difficult to accurately identify cases with early SDAT, it would be necessary to perform a longitudinal study using a large cross-section of the susceptible population (e.g. 70 to 80 year olds) and follow the levels of plasma AChE in these subjects. Retrospective analysis of enzyme levels of subjects who eventually develop SDAT will then be able to evaluate the usefulness of this enzyme as a marker of the disease. Also, a more promising approach in future



investigations may be the separation and analysis of the different molecular forms of AChE in plasma which may reveal more subtle underlying changes than the measurement of total enzyme activity.

More generally, since plasma AChE has an activity (approximately 7nmol/min/ml) of the same order as that found in CSF (approximately 20nmol/min/ml: Tower and McEachern, 1949; Johnson and Domino, 1971; Davies, 1979; Soininen et al 1981a; Wood et al, 1982; Appleyard et al, 1983; Arendt et al, 1984; Singer et al, 1984) the levels of this enzyme are worthy of further investigation both in SDAT and other disorders in which the levels of CSF AChE proved to be of limited use not only in SDAT (table 24), but also in other neuropsychiatric disorders (Davis and Goodnick, 1983).

### CONCLUDING REMARKS

In the present thesis, the cholinergic deficit in senile dementia of the Alzheimer type (SDAT) has been examined using cholinesterases as markers of cholinergic activity and the results obtained have implications for not only the aetiology but also the diagnosis and treatment of the disorder.

Analysis of the molecular forms of acetylcholinesterase (AChE) showed that the loss of activity of this enzyme in SDAT is due to the selective loss of the  $G_4$  molecular form in the cortex, but not nucleus of Meynert. The extensive loss of this form upon deafferentation of the rat hippocampus suggests that this form may be specifically associated with cholinergic synapses in the cortex. In future studies, the association of the  $G_4$  form of AChE with cholinergic synapses in the cortex could be examined using two different approaches: i) the laminar distribution of the molecular forms of AChE in the cortex could be determined and those forms associated with cholinergic synapses (i.e. with a distribution parallel to that of choline acetyltransferase) and non-cholinergic regions established; ii) synaptosomes could be prepared and the molecular forms associated with these isolated nerve terminals determined.

The observed specific loss of the  $G_4$  form of AChE has important implications for the experimental administration of anticholinesterases to SDAT subjects. Thus, the relatively moderate memory improvements observed in SDAT subjects upon administration of anticholinesterases such as physostigmine - which inhibits both AChE and butyrylcholinesterase (BChE) - may well be due to the limited amounts of drug that can be administered without risk of side effects. However, since the  $G_4$  form is present as a much smaller percentage of the total AChE in muscle (Carson et al, 1979) compared to the central nervous system (CNS), the use of a drug that specifically inhibits the  $G_4$  form of AChE would be relatively specific for nervous tissue and have few side effects in human muscle compared to an anticholinesterase such as physostigmine. Furthermore, such a drug, being a specific inhibitor of AChE, would not interact (unlike physostigmine) with plasma BChE, nor would it bind to erythrocyte AChE, which exists as the dimeric,  $G_2$  form (Ott et al, 1982). The effects, however, of a  $G_4$ -specific anti-AChE in the peripheral autonomic nervous system cannot be predicted since no reports exist describing the AChE molecular forms in these structures and therefore in future studies



it will be interesting to determine the AChE molecular forms in, for example, the human autonomic ganglia. In addition, a  $G_4$  specific anti-AChE would eliminate any possible side effects within the CNS due to the inhibition of BChE. Therefore, in summary, one could predict that the use of a drug that selectively inhibits the  $G_4$  form of AChE would greatly reduce the side effects of anticholinesterase administration. Consequently, larger doses could be administered to the patient and, since the amount of anticholinesterase that actually reaches the CNS appears to be related to the memory improvement observed (Thal et al, 1983), the therapeutic benefits of such a drug would be much greater than the anticholinesterases in current use. Whilst no anticholinesterase is currently recognised as being specific for the  $G_4$  form of AChE, little research has been carried out into the relative inhibition by anticholinesterases of the different AChE molecular forms and is therefore an area that merits future investigation.

The results presented here not only have important implications for the treatment of the memory loss in SDAT but they also suggest that not only may the cholinergic deficit in SDAT be not unique to demented Alzheimer-type subjects but it may also be unrelated to the cortical neuropathology of this disorder. Thus, analysis of the molecular forms of cholinesterases in demented Parkinsonian patients, in whom significant Alzheimer-type changes were absent, demonstrated a similar involvement to that seen in SDAT (i.e. a selective loss of the neocortical  $G_4$  form of AChE). Furthermore, the laminar distribution of cholinergic changes in SDAT cortex did not parallel the majority of neuropathological, morphological and neurochemical changes reported previously, suggesting that the loss of cortical cholinergic activity may be secondary to more fundamental pathological changes. It would therefore be interesting to analyse the laminar distribution of cholinergic changes in the demented Parkinsonian cortex as a further index of the similarity of the loss of cortical cholinergic activity in these two disorders. However, despite the similar loss of cortical cholinergic activity in demented Alzheimer-type and Parkinson's disease subjects, the involvement of the subcortical nucleus of Meynert may well be different. Thus, there is a much greater cell loss in this region in the Parkinson's disease compared to SDAT cases (Candy et al, 1983) which indicates that different pathological processes may be associated with the afferent cortical projections in these two disorders. Hence, in SDAT, loss of cortical cholinergic activity may be due to a retrograde loss of cholinergic



fibres from the nucleus of Meynert due to pathological changes in the cortex (i.e. the pathological changes occur primarily in the cortex). On the other hand, loss of cortical cholinergic activity in Parkinson's disease may be due to a loss of cell bodies in the nucleus of Meynert with a consequent loss of projecting fibres (i.e. the pathological changes occur primarily in the nucleus of Meynert). Therefore, it would be very interesting to determine the molecular forms of AChE in the nucleus of Meynert of demented Parkinsonian cases and compare them with those observed in SDAT.

In contrast to AChE, the analysis of both the molecular forms and laminar distribution of BChE suggest that there is no significant involvement of this enzyme in the neocortex in SDAT. Furthermore, the different distributions of this enzyme and cholinergic-related enzyme activities, both within the cortex and in different regions of the CNS, suggests that BChE is probably not associated with cholinergic neurotransmission in the CNS. In order to confirm this conclusion, it would be interesting to investigate the involvement of this enzyme in other diseases of the CNS in which the cholinergic system implicated (for example, amyotrophic lateral sclerosis). In addition, BChE should be investigated in other diseases of the CNS associated with different neurotransmitter systems (for example, the striatal dopaminergic system in Parkinson's disease) to determine whether or not BChE is associated with any other neurotransmitter.

More generally, the study of the intracortical distribution of neurochemical elements is a very useful technique for studying the specificity of neurochemical changes and should prove to be of interest not only in the further study of SDAT (for example the laminar distribution of changes associated with the noradrenergic and serotonergic systems) but also in other diseases marked by cortical biochemical (for example, alcoholic dementia) and neuropathological (for example, Pick's disease) changes.

The observation that plasma acetylcholinesterase levels are elevated in SDAT - but not Parkinson's disease - should encourage future studies of this enzyme not only in SDAT but in other disorders of the central nervous system, such as alcoholic dementia, in which the central cholinergic system is involved. To determine whether or not the increased level of plasma AChE observed in SDAT is derived from a disturbance of the blood-brain barrier, the levels of plasma AChE could be measured in diseases such as meningitis in which changes in the



blood-brain barrier occur. Alternatively, to determine whether or not degenerating cholinergic neurons are the cause of the increased plasma AChE levels (which might appear unlikely in view of the normal levels of plasma AChE observed in Parkinson's disease), plasma AChE could be measured in experimental animals in which central cholinergic pathways have been lesioned.

Whilst the results presented here confirm the loss of central cholinergic activity on SDAT, they also suggest that this deficit may not be a core feature associated with the characteristic Alzheimer-type changes. The measurement of other parameters found to be abnormal in SDAT (associated with, for example, the serotonergic and noradrenergic systems) in other diseases such as Parkinson's disease, should prove a useful means of distinguishing those changes that are and are not specific to SDAT and consequently help identify those changes that are most important in the underlying disease processes of the disorder.

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